Studies on the Internal Regulatory Mechanism of Genes Related to Ethylene Biosynthesis in Tomato Fruit

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Contents

Abbreviations used

ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
bp	basepair
CaMV	Cauliflower mosaic virus
cv	cultivar
DACP	Diazocyclopentadiene
GUS	ß-glucuronidase
kb	kilobase
LE	*Lycopersicon esculentum*
LUC	Luciferase
MCP	1-methylcyclopropene
MUG	4-methylumbeliferyl glucuronide
NBD	2,5-norbornadiene
NOS	Nopaline synthase
Nr	*Never ripe*
nt	nucleotide
SAM	S-adenosylmethionine

Chapter 1. General Introduction

1.1. *Ethylene biosynthesis in higher plants*

Ethylene, one of the simplest organic molecules with biological activity, is a plant hormone that regulates many aspects of plant growth, development and senescence (Theologis, 1992; Yang and Hoffman, 1984). All tissues in higher plants are capable of producing ethylene, although the production rate is normally low under steady states. However, endogenous ethylene production increases during certain stages of growth and development including seed germination, fruit ripening and leaves and flowers senescence and abscission. Ethylene production can also be induced by other external stimuli such as auxin, physical wounding, chilling injury, drought, water flooding and pathogen infection (Theologis, 1992; Yang and Hoffman, 1984). It has been recognized that this increased ethylene production can in turn bring about many important physiological consequences, such as fruit ripening, flower senescence, inhibition of growth, loss of geotropic sensitivity, onset of epinastic curvatures, acceleration of respiration, initiation of rooting, and modification of leaf and fruit pigments. (Oetiker and Yang, 1995; Theologis, 1992; Yang and Hoffman, 1984; Yang and Oetiker, 1998).

Ethylene has both positive- and negative- regulating effect on its own biosynthesis (Fluhr and Mattoo, 1996; Kende, 1993; Mattoo and White, 1991; Yang and Hoffman, 1984). Positive feedback regulation or autocatalysis of ethylene production is a characteristic feature of ripening fruits and other senescing tissues in which a massive increase in ethylene production is triggered by exposure to ethylene. Negative feedback regulation or autoinhibition of ethylene production has been recognized in a number of fruit and vegetative tissues (Yang and Hoffman, 1984). The availability of molecular probes for genes encoding the enzymes of ethylene biosynthesis and analysis of the

promoter sequences of these genes will undoubtedly aid in determining the mechanism of such feedback regulation (Kende, 1993). These feedback loops in ethylene biosynthesis have been shown to be able to uncouple by various inhibitors of ethylene action (Sisler et al., 1985; Wang and Woodson, 1989) or mutant plants for ethylene perception (Bleecker et al., 1988).

Ethylene in higher plants is synthesized via the following pathway: L-methionine → S-adenosyl –L-methionine (SAM) → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene (Adams and Yang, 1979). The enzymes catalyzing the individual step of this pathway are ACC synthase (EC 4.4.1.14., ACS) and ACC oxidase (EC 1.4.3., ACO). As many tissues are capable of converting applied ACC to ethylene, ACO activity is assumed to be constitutive (Yang and Hoffman, 1984). When high level of ethylene is produced, activity of ACS dramatically increases and that of ACO also is induced. It has been proposed that the conversion of SAM to ACC catalyzed by ACS and the oxidation step of ACC to ethylene by ACO are the late-limiting steps in ethylene biosynthesis (Fluhr and Mattoo, 1996; Theologis, 1992; Yang and Hoffman, 1984).

1.2.*Cloning of ACC synthase genes and their expression*

ACS protein has been purified and characterized from several plant tissues such as tomato (Bleecker et al., 1986; Van Der Straeten et al., 1989), winter squash (Nakajima et al., 1988), and apple (Dong et al., 1991b; Yip et al., 1991). By using their antibodies and library screening technique, their cDNAs have been cloned from zucchini (Huang et al., 1991; Sato and Theologis, 1989), winter squash (Nakajima et al., 1990), tomato (Lincoln et al., 1993; Olson et al., 1991; Rottmann et al., 1991; Van Der Straeten et al., 1990), and apple (Dong et al., 1991a). Seven regions of high homology among *ACS* genes have been identified and the most notable one among these conserved regions is the domain

around the active site of the enzyme. It has been pointed out that all known ACS contain, at comparable positions, 11 of 12 invariant amino acids that are involved in the binding of pyridoxal phosphate and substrate in any aminotransferases (Huang et al., 1991; Kende, 1993; Rottmann et al., 1991).

Expression of *ACS* gene has been investigated in several fruits including winter squash (Kubo et al., 1995; Mathooko et al., 1997; Nakagawa et al., 1991; Nakajima et al., 1990), zucchini (Huang et al., 1991), tomato (Li et al., 1992; Lincoln et al., 1993; Nakatsuka et al, 1997, 1998; Olson et al., 1991; Rottmann et al., 1991; Shiu et al., 1998; Spanu et al., 1993; Tatsuki and Mori, 1999; Tian et al., 1997; Van Der Straeten et al., 1990; Yip et al., 1992), apple (Dong et al., 1991b; Gorny and Kader, 1996, 1997; Sunako et al., 1999), melon (Bouquin et al., 1997; Shiomi et al., 1999b; Yamamoto et al., 1995), papaya (Mason and Botella, 1997), kiwi fruit (Ikoma et al., 1998, 1999; Whittaker et al., 1997; Xu et al., 1998), cucumber (Kamachi et al., 1997; Mathooko et al., 1999; Shiomi et al., 1998, 1999a; Trebitsh et al., 1997), passion fruit (Mita et al., 1998), Japanese pear (Itai et al., 1999), and banana (Liu et al., 1999). ACS is encoded by multigene family in these species and the expression of individual members has been shown to occur in different tissues and in response to specific stimuli known to induce ethylene biosynthesis (that is wounding, auxin, ripening and endogenous ethylene).

In tomato plant, ACS is encoded by at least nine genes (Kawakita and Theologis, unpublished; Zarembinski and Theologis, 1994). The marked increase of ACC and ethylene production can now be attributed to the expression of *ACS* genes *LE-ACS2* and *LE-ACS4* in ripening tomato fruit and accumulation of both transcripts is rapidly promoted by propylene application at mature green stage.

Genomic DNAs encoding ACS have also been cloned from several species such as zucchini (Huang et al., 1991), tomato (Lincoln et al., 1993; Olson et al., 1995; Rottmann et al., 1991; Shiu et al., 1998), and apple (Sunako et al., 1999).

On the basis of the introns present, *ACS* genes fall into three classes (Fluhr and Mattoo, 1996): *LE-ACS4* and *LE-ACS7* genes contain two introns, *LE-ACS2, LE-ACS3* and *LE-ACS6* genes contain three introns, however the four-intron gene has not yet been cloned from tomato fruit. Although *LE-ACS2* and *LE-ACS4* contain elements in their promoters that resemble an element in the ethylene inducible gene, *E4* in tomato (Cordes et al., 1989), the analysis of promoter activity using *ACS* gene 5′-flanking region has not been performed. Therefore, the *cis*-acting elements of *ACS* response to ethylene are still unclear.

1.3. *Cloning of ACC oxidase genes and their expression*

ACO was identified by a reverse genetic approach in tomato fruit (Hamilton et al., 1990) and subsequent identification of gene function by expression in yeast or oocytes of *Xenopus laevis* (Hamilton et al., 1991; Spanu et al., 1991). At present, numerous cDNA clones for ACO have been identified from many fruit species such as tomato (Hamilton et al., 1991; Holdsworth et al., 1987; Kock et al., 1991), avocado (McGarvey et al., 1990), apple (Dong et al., 1992; Ross et al., 1992), peach (Callahan et al., 1992), melon (Balague et al., 1993; Lasserre et al., 1996) and papaya (Lin et al., 1997). It has been demonstrated that ACO is encoded by multigene families and their cDNAs have highly conserved regions in the amino acid sequence of individual members. Expression of *ACO* genes have been investigated in fruits such as tomato (Barry et al., 1996; Holdsworth et al., 1988; Nakatsuka et al., 1997, 1998; Tian et al., 1997), avocado (McGarvey et al., 1992), peach (Callahan et al., 1992; Tonutti et al., 1997), apple (Gorny and Kader, 1996, 1997; Ross et al., 1992), melon (Balague et al., 1993; Bouquin et al., 1997; Lasserre et al., 1996; Shiomi et al., 1999b; Yamamoto et al., 1995), banana (Huang et al., 1997; Liu et al., 1999; Lopez-Gomez et al., 1997), kiwi (Ikoma et al., 1998, 1999; Whittaker et al., 1997; Xu et al., 1998), pear (Lelievre et al., 1997), passion fruit (Mita et al., 1998), and cucumber (Shiomi et al., 1998,

1999a). Their results show that the level of transcripts is stimulated by various stimuli, such as wounding, ripening, and exogenously applied ethylene. In addition, genomic sequences of *ACO* have been determined in melon (Lasserre et al., 1996, 1997), tomato (Blume et al., 1997a), banana (Huang et al., 1997; Lopez-Gomez et al., 1997) and apple (Atkinson et al., 1998) fruits, indicating that upstream regions of tomato *LE-ACO1* and melon *CM-ACO1* (that mainly express during fruit ripening) are very similar to sequences in the promoter of the tomato *E4* gene (Blume et al., 1997a; Lasserre et al., 1997). In transgenic tomato using *ACO* promoter-GUS fusions, *ACO* is regulated at the transcriptional level in a wide range of cell types at different developmental stages and the transcription responds to several external stimuli (Blume and Grierson, 1997b).

1.4. *Ethylene perception and signaling*

Unlike the ethylene biosynthetic pathway which is now clear, the mechanism of the ethylene action is just about to be unveiled owing to the recent isolation and epinastic studies of the ethylene response mutants from *Arabidopsis* (Ecker, 1995; Yang and Oetiker, 1998). Some components in the ethylene signaling cascade have been identified (Johnson and Ecker, 1998; Kieber, 1997). One of these genes, *ETR1*, shows similarity in deduced amino acid sequences to the prokaryotic two-component histidine kinases and most likely encodes an ethylene receptor. *CTR1* that locates in second cascade, encodes a protein with similarity to the ubiquitous Ras family of Ser/Thr protein kinases (Kieber et al., 1993). Activation of the *EIN3* family of nuclear proteins leads to induction of the relevant ethylene-responsive genes via other transcription factors, eliciting a response appropriate to the original stimulus.

1.4.1. *Cloning of ethylene receptor genes and their expression*

Bleecker et al. (1988) reported identification of the dominant *Etr1* ethylene-insensitive mutant of *Arabidopsis*. *ETR1* gene has been cloned by chromosome walking and shown to have sequence homology with bacterial two-component regulators (Chang et al., 1993). ETR1 protein forms membrane-associated dimers and, when expressed in yeast, binds ethylene (Schaller and Bleecker, 1995; Schaller et al., 1995). On the other hand, *ERS*, *ETR1* homolog, shares high degree of identity with the amino-terminal domain and putative histidine protein kinase domain of ETR1, but lacks the receiver domain (Hua et al., 1995). Recently, it was shown that *ETR1* is a member of a gene family consisting of five members: *ETR1*, *ERS*, *ETR2*, *EIN4*, and *ERS2* (Hua et al., 1995,1998; Sakai et al., 1998). In tomato fruit, *Never-ripe* (*Nr*) mutant is insensitive to ethylene (Lanahan et al., 1994) and a tomato locus linked *Nr* that hybridizes to the *Arabidopsis ETR1* gene was identified (Yen et al., 1995). Wilkinson et al. (1995) reported that *Nr* encodes a protein with homology to the *Arabidopsis* ethylene receptor *ETR1* but is lacking the response regulator domain as ERS-like protein and a single amino acid change in the sensor domain confers ethylene insensitivity. At present, five members of the tomato *ETR* gene family have been cloned (Lashbrook et al., 1998; Payton et al., 1996; Tieman and Klee, 1999; Wilkinson et al., 1995; Zhou et al., 1996). A number of *ETR1* homologous genes have been isolated from various fruits such as apple (Lee et al., 1998), *Citrus* (Li et al., 1998), passion fruit (Mita et al., 1998), and melon (Sato-Nara et al., 1999). These results show that ethylene receptor is also encoded by a multigene family.

Ethylene receptor gene families would be broadly expressed both spatially and temporally. The initiation of autocatalytic ethylene biosynthesis at the onset of ripening is correlated with a strong induction of *NR* mRNA at the breaker stage in tomato fruit (Lashbrook et al., 1998; Nakatsuka et al., 1998; Wilkinson et al., 1995). *LeETR4* is expressed at a very high level, accounting for more than

90% of the putative receptor expression in green tomato fruit and approximately 50% of the putative receptor expression in ripening fruit (Tieman and Klee, 1999). *ETR1* gene families are differentially regulated by ethylene. Although the expression of *ETR1* and *EIN4* was not appreciably affected by ethylene treatment in *Arabidopsis*, the RNA levels of the *ERS1*, *ETR2*, and *ERS2* genes were elevated in leaves by ethylene treatment (Chang et al., 1993; Hua et al., 1998).

1.4.2. *Ethylene response elements and their binding proteins*

Ethylene is known to exert its effects, at least in part, by altering gene expression. While effects of ethylene on both transcriptional and post-transcriptional processes have been shown (Lincoln and Fischer, 1988a), several additional genes have been identified that act downstream of *CTR1* (Ecker, 1995).

The activation of DNA-binding proteins involved in the regulation of particular genes represents the terminal step of ethylene signal transduction. Pathogen infection, senescence, and climacteric fruit ripening are ethylene-modulated processes that result in very different morphological and biochemical changes (Deikman, 1997; Johnson and Ecker, 1998). Excellent progress has been made in identifying the promoter elements necessary for ethylene-responsive transcription, and in studying the DNA-binding proteins that interact with these sequences (Deikman, 1997). Activation of plant defense genes in response to ethylene involves a promoter element called the GCC box, which interacts with ethylene-responsive element-binding proteins (EREBPs). One class of ethylene response element (ERE) is found in the upstream regions of genes induced during senescence in carnation (Itzhaki et al., 1994) and ripening in tomato fruit (Montgomery et al., 1993). The element shared among these genes is partially protected in footprinting assays by a DNA binding activity from carnation petal extracts (Itzhaki et al., 1994). Similarly, a nuclear factor from extracts of unripe

tomato interacts with this ERE upstream of the tomato *E4* gene (Montgomery et al., 1993). Two cooperative *cis*-elements are required for ethylene-responsive transcription of *E4* and *E8* genes during tomato fruit ripening. DNA-binding proteins of carnation and tomato that interact with ethylene-responsive *cis*-elements have been studied (Cordes et al., 1989; Coupe and Deikman, 1997; Deikman et al., 1998; Itzhaki et al., 1994; Maxson and Woodson, 1996; Xu et al., 1996), and in some cases cDNAs encoding such proteins have been isolated (Coupe and Deikman, 1997; Maxson and Woodson, 1996).

1.4.3. *Ethylene-regulated gene expression during fruit ripening*

Fruit ripening is associated with dramatic changes in gene expression (Gray et al., 1992; Ecker and Theologis, 1994). Among the five anonymous ripening-associated genes, *E4*, *E8*, *J49*, *E17*, and *D2*, only expression of*E4* is completely ethylene-dependent (Lincoln et al., 1987). The others are either developmental or ethylene-regulated, and their expression is quite complex (Theologis et al., 1993). Surprisingly, *E4* gene expression is not restored in *LE-ACS2* antisense fruits by treatment with propylene, an ethylene analog. *E8* gene expression has been reported to be ethylene-regulated, but ethylene-independent feature of *E8* has also been shown (Theologis et al., 1993). Recently, it was shown that transgenic tomato fruit expressing antisense *E8* mRNA produces ten-fold higher levels of ethylene (Penarrubia et al., 1992), indicating that E8 protein is a negative regulator of ethylene biosynthesis.

With the progress in research on molecular aspects of ethylene biosynthesis as a background information, the present work focuses on understanding of the internal feedback regulation of ethylene biosynthesis at the transcriptional level in tomato fruit. The objectives of this study include; a) understanding the positive and negative regulatory mechanisms of ethylene during development

and ripening, b) identification of ethylene-responsive *cis*-elements in *ACS* promoter region, and c) understanding the relationship between the expression of *E4* and *E8* genes and ethylene.

Chapter 2. Internal feedback regulation of ethylene biosynthesis in tomato fruit

2.1. Positive feedback regulation of ACC synthase and ACC oxidase genes

Introduction

It is well known that ethylene biosynthesis is subject to both positive and negative feedback regulation (Kende, 1993). Positive feedback regulation of ethylene biosynthesis is a characteristic feature of ripening fruits and senescing flowers. In tomato and cantaloupe fruits (Liu et al., 1985), banana fruit (Inaba and Nakamura, 1986), and carnation (Wang and Woodson, 1989) and morning glory (Suttle and Kende, 1980) flowers, a massive increase in ethylene production is triggered by exposure to exogenous ethylene with activation of ACS and/or ACO. Negative feedback has been recognized in a number of fruit and vegetative tissues. In *Citrus* fruit discs (Riov and Yang, 1982), banana fruit tissue (Vendrell and McGlasson, 1971), fig fruit (Zeroni et al., 1976), winter squash discs (Hyodo et al., 1985), tobacco leaf (Aharoni et al., 1979), and mung bean hypocotyls (Yoshii and Imaseki, 1982), exogenous ethylene significantly inhibits endogenous ethylene production induced by ripening, wounding, and/or treatment with auxin.

With the advances in molecular cloning techniques, it has been demonstrated that both ACS and ACO are encoded by a multigene family in various plant organs (Kende, 1993). In tomato plant, at least nine genes encode ACS (Kawakita and Theologis, unpublished; Zarembinski and Theologis, 1994); ACO is encoded by three (Barry et al., 1996). These genes have been isolated and structurally characterized with different expressions in various tissues at different stages of development and in response to specific stimuli, which induce ethylene biosynthesis. Among these genes, two for *ACS*, *LE-ACS2* and *LE-ACS4*

cloned by Rottmann et al. (1991) and Lincoln et al. (1993), and one for *ACO*, *LE-ACO1* cloned by Barry et al. (1996), are the genes transcripted during fruit ripening concomitant with ethylene biosynthesis. Tomato fruit exhibits a climacteric rise of respiration with a concomitant burst in ethylene production, which is also induced by treatment with exogenous ethylene (Biale and Young, 1981). In preclimacteric tomato fruit exposed to ethylene for 48 h, the expression of *LE-ACS2* and *LE-ACS4* genes is induced in a dose-dependent manner (Lincoln et al., 1993; Rottmann et al., 1991), demonstrating an involvement of a positive feedback regulation at the transcriptional level in these two genes. Liu et al. (1985) reported that the low level of ACO activity in mature green tomato fruit is increased markedly by ethylene treatment in dose- and time-dependent manner and that this increase is inhibited by NBD, an ethylene action inhibitor. Based on the above mentioned fact that only one gene for *ACO* is mainly expressed in ripening tomato fruit, it is probable that *ACO* gene expression is also regulated under a positive feedback system. Therefore, the expression of both *ACS* and *ACO* genes are probably regulated by a positive feedback induced by the ethylene produced by tomato fruit. However, this concept is based on the study with preclimacteric fruit, and it has not yet been clarified whether or not the same regulation system operates in the fruit even after the burst of ethylene production has commenced.

In this section, using 1-methylcyclopene (MCP), a new inhibitor of ethylene action (Serek et al., 1994), we demonstrate that the regulation and expression in tomato fruit of the *ACS* and *ACO* genes mentioned above is under a positive feedback control mechanism even at the stage of massive ethylene production.

Materials and Methods

Plant materials

Greenhouse grown tomato (*Lycopersicon esculentum* Mill. cv. TVR-2, a popular and normal ripening variety in Japan) fruit were harvested at mature green and turning stages from a commercial farm. Mature green fruit were treated with 2,000 μl•liter^{-1} propylene in an enclosed 40-liter chamber for 24 h and then ripened at 20℃ for 2 days. Ethylene production was monitored every day. Pericarp tissues from the equatorial region were frozen in liquid nitrogen and stored at -80℃ until RNA extraction. Turning fruit were ripened at 20℃ for 6 days. During ripening, fruits were treated with MCP at or 2 days after (pink stage) harvest. The rate of ethylene production from whole fruit and red color development on the equatorial fruit surface were measured after every 2 days. Pericarp tissues from the equatorial region were frozen in liquid nitrogen and stored at -80℃ until extraction of total RNA, ACC, ACS, and ACO. Color measurements were made using a color difference meter (Model 1000DP, Nippon Denshoku Kogyo, Tokyo). The 'a' value on the lab scale was used as a continuous scale, negative for green color and positive for red.

MCP synthesis and treatments

MCP was synthesized according to the method of Magid et al. (1971) as stable lithium derivative in ether solution and stored at -20℃ until use. A small amount of ether solution was put in a small test tube with a rubber stopper, and then MCP gas was generated by aqueous neutralization of the lithium derivative. Fruit samples were sealed in a 10-liter glass jar fitted with a rubber stopper. Using hypodermic syringe, the headspace gas in the test tube containing MCP gas was withdrawn and injected into the jars containing the fruit. The jars were then incubated for 6 h at room temperature. The concentration of MCP in the jar

was estimated to be in the range of 10 to 20 nl•liter^{-1}.

Inhibitory effect of MCP on ethylene action

To examine the nature of the inhibitory effect of MCP on ethylene action, mature green fruit treated with MCP were exposed to 1,000 μl•liter^{-1} ethylene for 24 h, and then ethylene production from fruit was monitored every day.

Ethylene biosynthesis

Ethylene production was measured by enclosing fruit samples in an airtight chamber for 1 h at 20℃, withdrawing 1 ml of head space gas from the chamber, and injecting it into a gas chromatograph fitted with a flame ionization detector and an activated alumina column. ACC was measured by the method of Lizada and Yang (1979), with 80% ethanol extracts from frozen pericarp tissues which were partially purified on cation-exchange resin [Amberlite CG-120 (H^+)] column. Both ACS and ACO were extracted using the same buffer. Five grams of frozen pericarp tissue was homogenized with 10 ml of extraction buffer consisting of 500 mM potassium phosphate (pH 8.5), 30 mM sodium ascorbate, 5 mM DTT, 5 μM pyridoxal phosphate, 2% PVP, and 10% glycerol. To obtain the enzyme solution, the homogenate was filtered through four layers of cheesecloth and centrifuged at 30,000 xg for 20 min. The obtained supernatant was desalted by passage through a Sephadex G-25 column previously equilibrated with the elution buffer consisting of 100 mM potassium phosphate (pH 8.5), 30 mM sodium ascorbate, 5 mM DTT, 5 μM pyridoxal phosphate, and 10% glycerol. All steps in enzyme extraction were done at 4℃. ACS activity was assayed by incubating 1 ml of the enzyme preparation with 0.2 ml of 0.5 mM SAM at 30℃ for 30 min, and the ACC produced was determined. The enzyme activity was expressed as the amount of ACC (nmol) produced per mg of protein per hour. ACO activity was assayed by incubating 1 ml of the enzyme preparation with 0.1

ml of 20 mM ACC, 0.01 ml of 2 mM $FeSO_4$, and 0.2 ml of 300 mM $NaHCO_3$ at 30℃ for 30 min, and the ethylene produced was determined. The enzyme activity was expressed as the amount of ethylene (nmol) produced per mg of protein per hour. Protein content in the enzyme extracts was estimated by the dye-binding method of Bradford (1976) using bovine serum albumin as a standard.

RNA extraction, and isolation and amplification of poly (A)$^+$ RNA

RNA was at first extracted by the SDS-phenol method (Sambrook et al., 1989) with minor modification and later by the hot borate method (Wan and Wilkins, 1994). Poly (A)$^+$ RNA was isolated using oligo dT (Takara, Kyoto) according to the manufacture's protocol. The first strand cDNAs synthesized by reverse transcriptase from 2 μg of the poly (A)$^+$ RNA isolated from ripe tomato fruit were amplified by the RT-PCR method using mixed oligonucleotide primers A and B for *ACS* (*LE-ACS2* and *LE-ACS4)* and primers C and D for *ACO* (*LE-ACO1*) as shown in Table 2.1.1. These primers were synthesized with reference to the conserved amino acid sequences of *ACS* [conserved regions 1 and 7, Kende (1993)] and *ACO* [amino acid residues 39-45 and 297-302, Kende (1993)] with restriction-site sequences of *Sph* Ⅰ and *Sal* Ⅰ (indicated in parenthesis in Table 2.1.1). Reactions were subjected to 25 cycles of 94℃ for 1 min, 37℃ for 2 min, and 72℃ for 3 min.

Cloning and screening the PCR products

After washing with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitation with ethanol, PCR products were digested with *Sph* Ⅰ and *Sal* Ⅰ, and ligated into the corresponding sites in pUC118 plasmids (Takara, Kyoto). *E. coli* MV1184 was transformed with the ligation mixture, plated onto blue-white selection plates, and incubated overnight at 37℃. Plasmids were isolated from

Table 2.1.1. Oligonucleotide primers used for amplification of cDNAs by RT-PCR

	Name	DNA sequence	gene
A	ACS-F	5´-cc(gcatgc)tgggtytngcwgaraatcagct-3´	degenerate
B	ACS-R	5´-gg(gtcgac)arcaaacwcgraaccamcctgg-3´	
C	ACO-F	5´-cccc(gcatgc)saraaytggggyttstwygag-3´	
D	ACO-R	5´-gggg(gtcgac)tcraabckyggytcyttng-3´	

recombinant white colonies and digested with various restriction enzymes to screen colonies having respective cDNAs possibly encoding ACS and ACO. Double-stranded plasmid cDNAs isolated from colonies with different size and restriction-site sequences was sequenced using 373A (Applied Biosystems) or DSQ-1000 (Shimadzu) DNA sequencers using either the -21M13 or M13 sequencing primers according to the manufacture's instructions (Amersham).

RNA blotting and hybridization

Three-micrograms of mRNA samples isolated from pericarp tissues were separated by electrophoresis on 1% agarose gels containing 0.66 M formaldehyde, blotted onto nylon membranes (Hybond N, Amersham), and fixed by heating at 80℃ for 2 h. Membranes were prehybridized at 42℃ for 2 h in a solution containing 50% formamide (v/v), 5 x Denhardt's reagent (1 x Denhardt's solution is 0.02% each of Ficoll-400, PVP and BSA), 0.1% SDS, 5 x SSPE [1 x SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA (pH 7.4)], and 100 $\mu g \cdot ml^{-1}$ denatured fragmented herring sperm DNA. Hybridization was performed overnight in an identical buffer solution containing 5 x 10^5 $cpm \cdot ml^{-1}$ denatured ^{32}P-labeled cDNA probes (*tACS2*, *tACS4* and *tACO*) corresponding to *LE-ACS2*, *LE-ACS4*, and *LE-ACO1*. cDNA probes were labeled by random primed DNA labeling kit (Boehringer Mannheim) with [^{32}P]dCTP at > 100 $TBq \cdot mmol^{-1}$. Following hybridization, membranes were washed twice at 60℃ in 2 x SSPE and 0.1% SDS for 30 min and subsequently exposed to an imaging plate (Fuji Photo Film, Tokyo) at room temperature. Cross-hybridization between *LE-ACS2* and *LE-ACS4* was not observed in slot blot analysis under these stringent conditions. Equal mRNA loading was confirmed by rehybridization with a 0.4 kb actin cDNA after stripping off the former probes by washing the membranes in 0.1% SDS solution at 80℃ for 30 min.

Results

Isolation and identification of cDNA clones

By using mixed primers designed from conserved amino acid sequences among already identified *ACS* and *ACO* from various plant organs, we cloned two cDNA fragments for *ACS*, *tACS2* and *tACS4*, and one cDNA fragment for *ACO*, *tACO*, from RNA extracted from ripened tomato fruit. The *tACS2* and *tACS4* showed high degrees of sequence similarity to *LE-ACS2* (Rottmann et al., 1991) and *LE-ACS4* (Lincoln et al., 1993), respectively, with up to 99% homology at the nucleotide level in both cDNAs. The *tACO* displayed high similarity to *LE-ACO1* (Barry et al., 1996) in the sequence with 99% identity at the nucleotide level. Each of the cloned cDNA fragments also had conserved amino acid sequences for *ACS* and *ACO*. Comparative analysis of the cloned cDNA to each corresponding cDNA registered on EMBL database revealed the following characteristics: *tACS2*, coding 264-1,311 bp of *LE-ACS2* (accession no X59145) with two changes (C to T) at nucleotides 461 and 1,297 on the cDNA sequence; *tACS4*, coding 237-1,282 bp of *LE-ACS4* (accession no X59146) with a single change (G to A) at 292; *tACO*, coding 203-983 bp of *LE-ACO1* (accession no X58273) with a single change (A to T) at 811. For the reason mentioned above, we considered the cDNAs to be fragments of the same *ACS* and *ACO* genes. These fragments were used as a probe in northern blot analysis.

Effect of propylene on gene expression

To confirm the characteristics of positive feedback expression of the respective three genes, *LE-ACS2* and *LE-ACS4* for *ACS* and *LE-ACO1* for *ACO*, mature green tomato fruit were treated with 2000 μl•liter^{-1} propylene for 24 h at 20℃. Ethylene production was induced 2 days after treatment (data not shown) with concomitant increase in *LE-ACS2* and *LE-ACS4* mRNA abundance (Fig.

2.1.1.), indicating a positive feedback regulation of these two *ACS* genes at the onset of climacteric. However, propylene treatment had little effect on the expression of *LE-ACO1*, indicating that this gene is already expressed abundance in mature green tomato fruit.

Inhibitory effect of MCP on ethylene action

Figure 2.1.2. shows induction of endogenous ethylene production by exogenous ethylene in mature green tomato fruit treated with or without MCP. Exogenously applied ethylene stimulated endogenous ethylene production immediately after the application. MCP almost completely eliminated this stimulative action of exogenous ethylene even at a concentration much higher than the endogenous level.

Effect of MCP on red color development

Figure 2.1.3. shows red color development in tomato fruit which were harvested at turning stage and treated with or without MCP (control) at turning or pink stages followed by ripening at 20℃. In the control fruit, red color developed normally, reaching pink, red, and full-ripe stages in 2, 4, and 6 days after harvest, respectively. MCP almost completely suppressed red color development in the fruit exposed at the turning stage throughout experimental period, indicating a strong irreversible binding to the ethylene receptor site. In the fruit treated with MCP at pink stage, red color development was only slightly inhibited.

Effect of MCP on ethylene biosynthesis

Ethylene production in turning fruit increased during ripening, reaching a peak at 4 days (Fig. 2.1.4A). The production was reduced by about 50% for 4 days by MCP treatment both at turning or pink stages. However, in fruit and

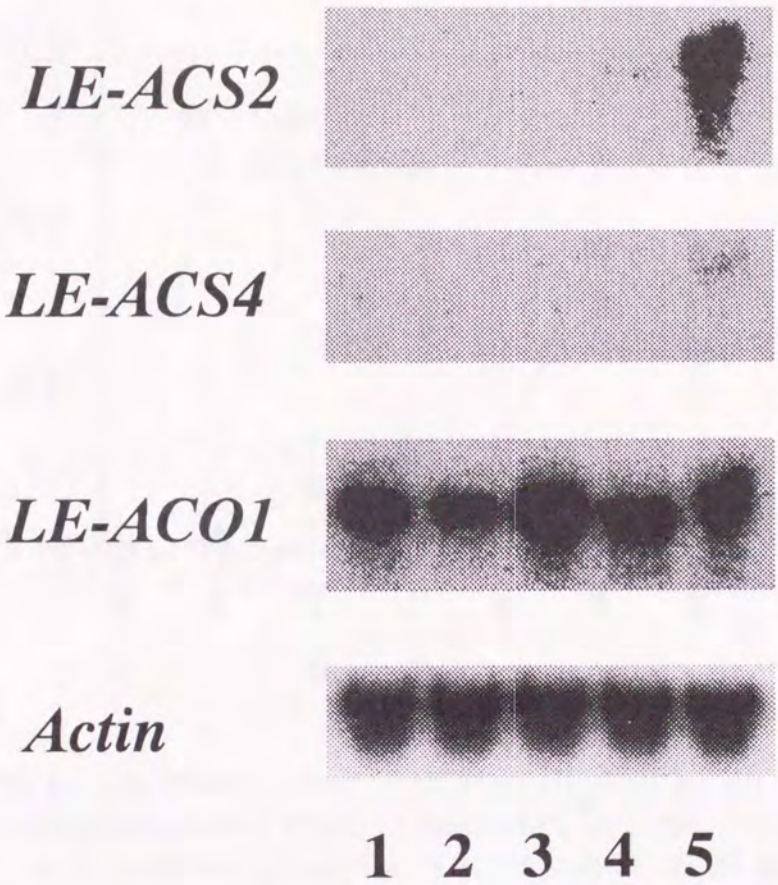


Figure 2.1.1. Effect of propylene on the accumulation of *LE-ACS2*, *LE-ACS4,* and *LE-ACO1* mRNAs in mature green fruit. Lane 1, control fruit at harvest; lane 2, control fruit 2 days after harvest; lane 3, control fruit 4 days after harvest; lane 4, propylene-treated fruit for 2 days; lane 5, propylene-treated fruit for 4 days. Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amount of mRNA loaded.

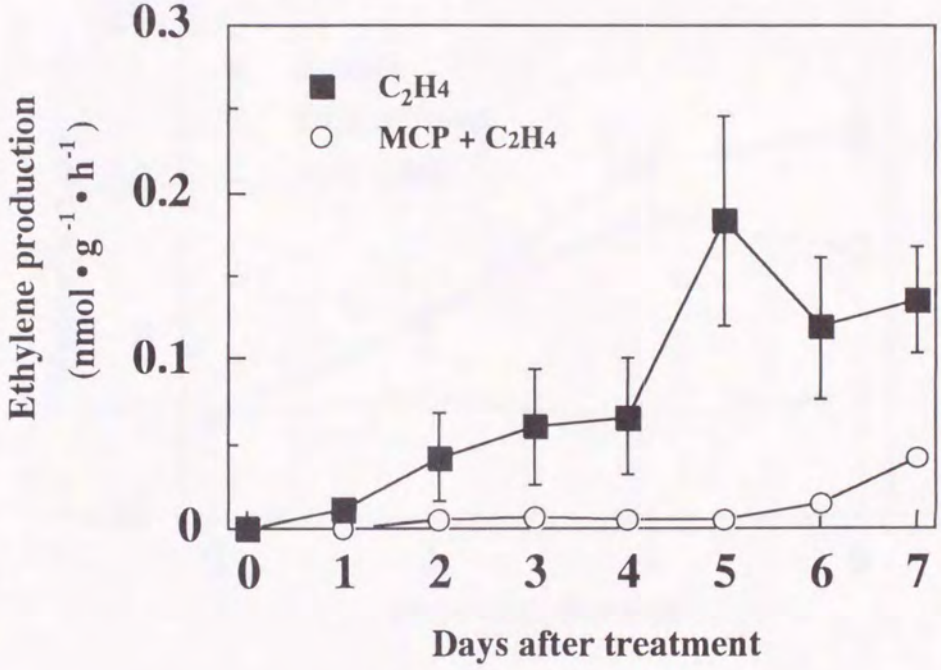


Figure 2.1.2. Inhibitory effect of MCP on ethylene action in the induction of endogenous ethylene production in tomato fruit. Mature green fruit were treated with or without 10 to 20 nl•liter^{-1} MCP for 6 h followed by treatment with 1,000 μl•liter^{-1} ethylene for 24 h and then ripened at 20℃. Vertical bars are the SE of three replications. When absent, the SE bars fall within the dimensions of the symbol.

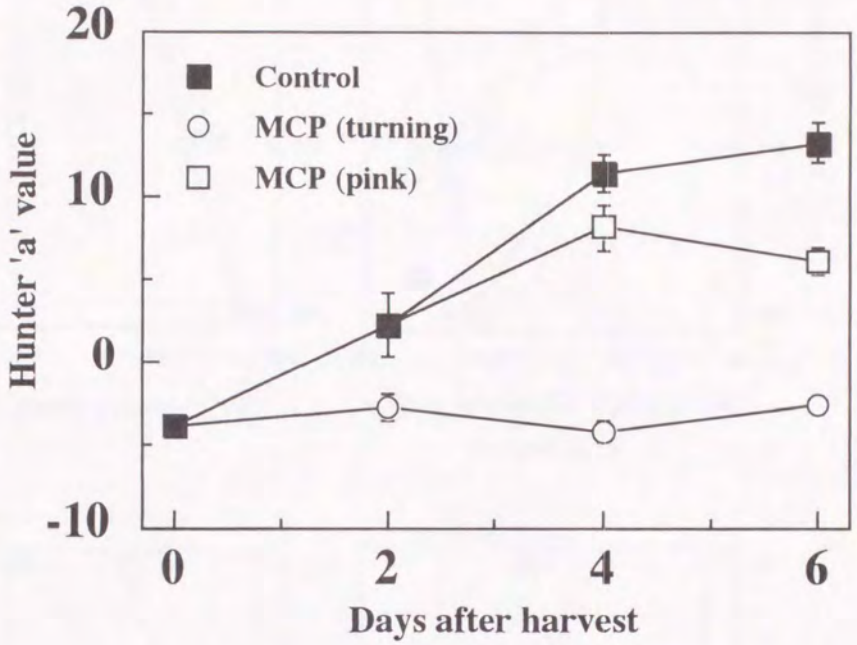


Figure 2.1.3. Effect of MCP on red color development in the fruit during ripening. Fruits were harvested at turning stages and treated with 10 to 20 nl•liter^{-1} MCP for 6 h at turning (at harvest) or pink (2 days after harvest) stages. Vertical bars are the SE of five replications. When absent, the SE bars fall within the dimensions of the symbol.

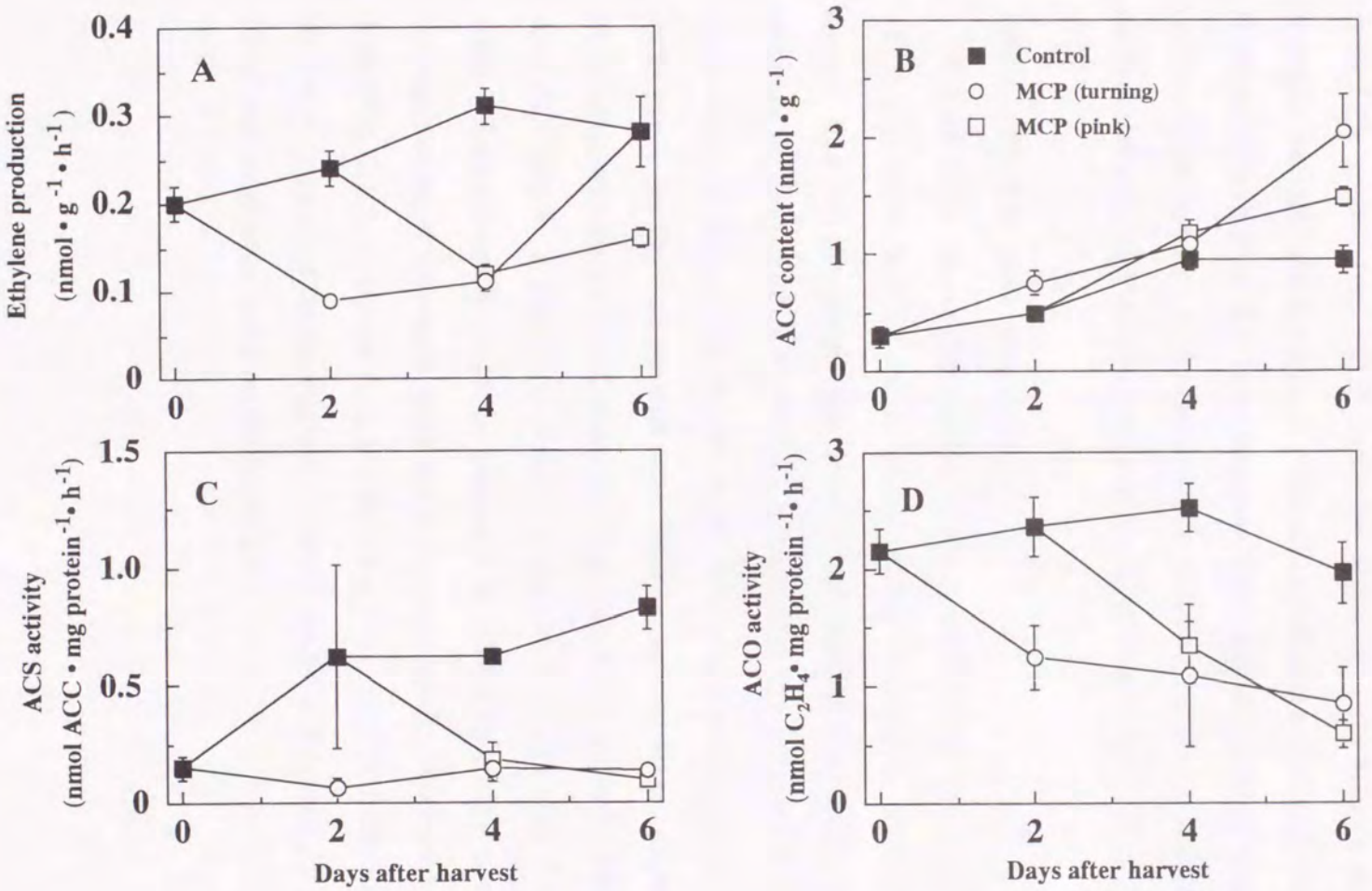


Figure 2.1.4. Effect of MCP on the rate of ethylene production (A), ACC content (B), and activities of ACS (C) and ACO (D) in tomato fruit during ripening. Harvest stage and MCP treatment were the same as in Figure 2.1.3. Vertical bars are the SE of three replications. When absent, the SE bars fall within the dimensions of the symbol.

treated with MCP at turning stage, ethylene production at day six was similar to that of the control fruit. MCP treatment of fruit at both turning and pink stages strongly inhibited the increase in ACS activity that is associated with the onset of ripening in fruit (Fig. 2.1.4C). However, ACC content in MCP treated fruit was rather higher than that in the control (Fig. 2.1.4B). MCP also strongly suppressed ACO activity in fruit at both stages of ripening (Fig. 2.1.4D).

Effect of MCP on gene expression

Figure 2.1.5. shows the inhibitory effect of MCP on the expression of *LE-ACS2*, *LE-ACS4*, and *LE-ACO1* genes during ripening of tomato fruit. In the control fruit, all the genes were already expressed at the turning stage, and the amounts of their mRNA transcripts increased at the pink stage followed by a slight decrease toward the red stage. MCP strongly inhibited the expression of these genes. In the fruit treated with MCP at turning stage, expression of *LE-ACS2* gene was almost eliminated for 2 days with a slight increase in the next 2 days followed by a great recovery in further next 2 days (Fig. 2.1.5., compare lanes 2-3 with lanes 4-6). In the treatment at pink stage, MCP greatly inhibited the expression of *LE-ACS2* gene for 2 days; it rapidly recovered in the next 2 days (Fig. 2.1.5., compare lane 3 with lanes 7-8). Similar inhibitory patterns of MCP were observed on the expression of *LE-ACS4* and *LE-ACO1* genes, but the effect was somewhat weak on the latter gene.

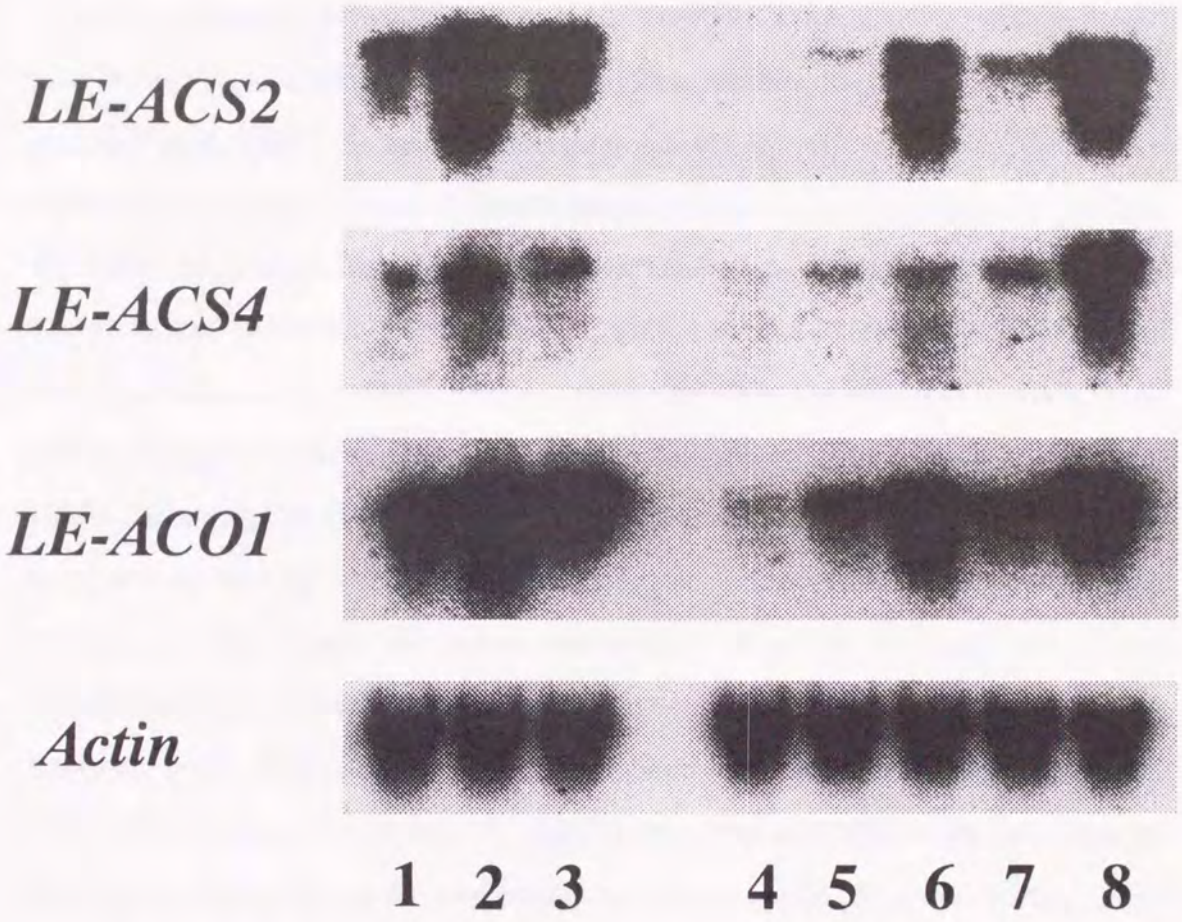


Figure 2.1.5. Effect of MCP on the accumulation of *LE-ACS2*, *LE-ACS4*, and *LE-ACO1* mRNAs in turning and pink fruits. Harvest stage and MCP treatment were the same as in Figure 2.2. Lane 1, control fruit at harvest; lane 2, control fruit 2 days after harvest; lane 3, control fruit 4 days after harvest; lane 4, turning-stage fruit 2 days after MCP treatment; lane 5, turning-stage fruit 4 days after MCP treatment; lane 6, turning-stage fruit 6 days after MCP treatment; lane 7, pink-stage fruit 2 days after MCP treatment; and lane 8, pink-stage fruit 4 days after MCP treatment. Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amount of mRNA loaded.

Discussion

Ethylene has been shown to regulate its own biosynthesis in the two opposite directions. In positive feedback regulation, ethylene stimulates its own synthesis, and in negative feedback regulation, ethylene inhibits its own synthesis (Yang and Hoffman, 1984). At the gene expression level for ACS and/or ACO, the two key enzymes in the ethylene biosynthetic pathway, the involvement of a positive feedback regulation in ethylene biosynthesis has been elucidated in the senescence of carnation (Woodson et al., 1992), orchid (O'Neil et al., 1993), and petunia (Tang and Woodson, 1996) flowers, ripening tomato fruit (Lincoln et al., 1993), mung bean (Kim and Yang, 1994) and pea seedlings (Peck and Kende, 1995). In carnation (Woodson et al., 1992) and orchid (O'Neil et al., 1993), exogenously applied ethylene induces the expression of *ACS* and *ACO* genes in a manner similar to that of flower senescence. However, the increase in the abundance of *ACS* and/or *ACO* mRNAs in senescing flowers is prevented by treatment with NBD. Similar results have been reported for seedlings, in which exogenous ethylene counteracts the inhibitory action of NBD on the induction of *ACO* gene expression in excised mung bean hypocotyls (Kim and Yang, 1994) and in auxin treated pea epicotyls (Peck and Kende, 1995). In tomato, it has been demonstrated that exposure of mature green fruit to exogenous ethylene induces transcription of *ACS* genes in a dose-dependent manner similar to that found during natural fruit ripening (Lincoln et al., 1993), although there are no data available with respect to ethylene action inhibitors. It has been shown that treatment of tomato and cantaloupe fruits with ethylene for 16 h markedly increases ACO activity without any increase in ACC content or in ethylene production rate (Liu et al., 1985). These findings suggest that ethylene biosynthesis is under positive feedback regulation during flower senescence and fruit ripening. However, a question remains whether positive feedback regulation

of ethylene biosynthesis truly operates in the fruit which have already reached to climacteric peak, because all findings mentioned above are obtained from preclimacteric organs.

The present study clearly demonstrates that a strong positive feedback regulation is involved in ethylene biosynthesis in the tomato fruit even at the stage of a massive ethylene production. MCP completely suppressed red color development when applied to fruit at turning stage and inhibited only slightly when applied to fruit at pink stage. Similar results were obtained in tomato fruit which were exposed to DACP, another recently developed compound that blocks ethylene action (Sisler and Lallu, 1994). Serek et al. (1994, 1995) have previously reported a strong effect of MCP in blocking ethylene action in several potted and cut flowers. This effect was a function of concentration and time of exposure, in which a very low binding constant ($Kd=8nl \cdot liter^{-1}$) was obtained in a competition assay between MCP and ^{14}C-ethylene. Cycloolefine compounds such as NBD and DACP have been shown to block various actions of ethylene in many plant organs (Abeles et al., 1992). In the present study, MCP completely suppressed ethylene action with respect to induction of endogenous ethylene production in mature green tomato fruit. Induction of ethylene production in climacteric fruits is one of the typical responses to exogenous ethylene treatment. Our results strongly indicate that MCP may act effectively in blocking ethylene action in tomato fruit. Ethylene production from intact tomato fruit at the turning and pink stages was also greatly reduced by MCP treatment. This reduction agrees well with the observed suppression of both ACS and ACO activities in the fruit treated with MCP. Sisler and Lallu (1994) reported a suppression of ethylene production in tomato fruit treated with DACP, although this suppression was less than in our present results using MCP. However, MCP treatment seemed to have little effect on ACC content. This may be due to the fact that MCP suppresses ACO activity with the result that little ACC is converted to ethylene,

and, therefore ACC content tends to remain at the control level.

The nucleotide sequences of the two probes for *ACS* (*LE-ACS2* and *LE-ACS4*) and one probe for *ACO* (*LE-ACO1*) genes obtained in the present study had very high homology to those previously identified in tomato fruit (Rottmann et al., 1991; Lincoln et al., 1993; Barry et al., 1996). The expression of three genes decreased by MCP treatment at either turning or pink stages for 2 to 4 days. This is especially so in the fruit treated with MCP at turning stage, where the abundance of *LE-ACS2* mRNA was completely eliminated 2 days after treatment, the stage when the control fruit were at pink stage with the greatest mRNA accumulation. Abundance of mRNA of *LE-ACS2* was also markedly decreased in the fruit treated with MCP at pink stage. We also observed great reduction in the levels of mRNA for *LE-ACS4* and *LE-ACO1* with MCP treatment. Among at least nine *ACS* divergent genes in tomato plant, two of them, *LE-ACS2* and *LE-ACS4*, have been demonstrated to be expressed during fruit ripening (Lincoln et al., 1993; Rottmann et al., 1991). In addition, the expression of these genes has been shown to be regulated under a positive feedback mechanism in ethylene-treated mature green fruit. For *ACO*, three genes have been identified in tomato plant (Barry et al., 1996). One of them, *LE-ACO1* is expressed throughout the duration of ripening with a marked increase in the transcript at the breaker stage from a low basal level. The positive feedback regulation of this *ACO* gene expression has not yet been clarified, but there is evidence that ACO is activated by ethylene in various plant organs. The results show that a short time exposure of preclimacteric tomato fruit to ethylene markedly increases its capability to convert ACC to ethylene (Liu et al., 1985), coinciding with the pattern of expression of the *LE-ACO1* gene mentioned above (Barry et al., 1996) and suggesting an involvement of a positive feedback regulation mechanism in expression of this gene at the onset of climacteric rise. The present results clearly demonstrate that expression of all the three genes examined is highly regulated

under a positive feedback mechanism in tomato fruit, at both the stage when massive increase in ethylene production has commenced as well as at the onset of the climacteric rise.

The signals in the MCP treated fruit recovered to the control level within 6 days in the turning and 4 days in the pink fruits. This recovery may indicate either the production of new ethylene receptor sites or release of the MCP from already existing receptor sites. The former concept may be more likely since elevation in the level of mRNA which is structurally similar to the *ERS*, a kind of ethylene receptor gene, in ripening tomato fruit has already been demonstrated (Wilkinson et al., 1995).

Elucidation of the negative feedback regulation of ethylene biosynthesis at the level of gene expression is limited, with the exception reported by Nakajima et al. (1990), in which the accumulation of translatable mRNA against wound-induced *ACS* in winter squash fruit was suppressed by ethylene and stimulated by NBD. In the present study, although expression of each mRNA examined was strongly suppressed for 2 days after MCP exposure to turning fruit, ethylene biosynthesis in the same fruit was not inhibited to the level expected with respect to suppression of the gene expression. This contradiction may suggest that other mRNA(s), whose expression is suppressed under natural fruit ripening by the burst of ethylene production, and is induced in the fruit treated with MCP as a result of blocking their negative feedback regulation. It may also be possible that the enzymes for ethylene biosynthesis still exist in the cell, although their gene expression had already been eliminated. However, a much shorter turnover time of ACS has been demonstrated in plant organs with inactivation feature by its substrate, SAM (Satoh and Yang, 1988). In the tomato plant, four *ACS* genes are known to be differentially expressed in response to developmental, environmental, and hormonal factors in different organs (Yip et al., 1992). Similar differential expressions of three genes encoding ACO have been

demonstrated in different tomato organs in which, unlike *LE-ACO1*, the *LE-ACO3* transcript appeared only transiently in the fruit at breaker stage (Barry et al., 1996). This transient appearance may indicate a negative feedback feature of *LE-ACO3* gene at the stage of massive ethylene production.

The data presented in this section indicate a positive feedback regulation for the expression of both *ACS* and *ACO* genes during fruit ripening, at both the stage of massive ethylene production and at the stage of climacteric onset, the latter in the already known manner. The regulation of *ACS* and *ACO* genes by ethylene during ripening, including the existence of negative feedback regulation will be the focus of our future experiments.

Summary

We have examined whether or not a positive feedback regulation of gene expression for *ACS* and *ACO* also operates in ripening tomato (*Lycopersicon esculentum*) fruit during the burst of ethylene production. Two cDNA fragments for *ACS* (*LE-ACS2* and *LE-ACS4*) and one for *ACO* (*LE-ACO1*) were cloned with high homology to already known genes involved in ethylene biosynthesis in ripening tomato fruit. Accumulation of two *LE-ACS* transcripts was induced in mature green fruit within 2 days by treatment with propylene. In the fruit harvested at the turning stage, red color development, ethylene production, ACC content, and activities of ACS and ACO increased as maturity progressed. The expression of *LE-ACS2*, *LE-ACS4* and *LE-ACO1* in the fruit increased from the turning to pink stage and were followed by a slight decline towards the red stage. These increases in mRNAs abundance with ripening were prevented to a large extent by treatment with the ethylene action inhibitor, MCP. This was mostly pronounced in the fruit treated with MCP at turning stage, in which the accumulation of *LE-ACS2*, *LE-ACS4* and *LE-ACO1* transcripts was almost completely eliminated in the first 2 days, precisely the same stage at which the control fruit had the greatest level of each mRNA accumulation. The inhibition of transcript accumulation recovered to the control level within 2 to 4 days. MCP also decreased ethylene biosynthetic activity, although this decrease did not reflect the reduction in the mRNAs accumulation. These results suggest that a strong positive feedback regulation is involved in ethylene biosynthesis at the gene transcriptional level in tomato fruit, even at the stage of a burst in ethylene production.

2.2. Positive and negative feedback regulation of genes related to ethylene biosynthesis

Introduction

Fruits can be classified as climacteric or nonclimacteric depending on the presence or absence of massive ethylene production during ripening and on their response to exogenous ethylene (Biale and Young, 1981). Even in climacteric fruit, ethylene production is generally very low until the commencement of ripening. At the onset of ripening, fruit exhibit a climacteric increase in respiration, with a concomitant burst of ethylene production. Based on the level of ethylene production during fruit development, McMurchie et al. (1972) introduced the concept of system 1 and system 2 ethylene. System 1 is the basal low rate of ethylene production present in preclimacteric fruits. The basal level of ethylene produced by vegetative tissues and nonclimacteric fruits can be classified as system 1 (Oetiker and Yang, 1995). On the other hand, system 2 is the high rate of ethylene production observed during ripening in climacteric fruits and in certain senescent flowers (Oetiker and Yang, 1995). As mentioned in section 2.1., ethylene biosynthesis is subject to both positive and negative feedback regulation (Kende, 1993). In tomato (*Lycopersicon esculentum*) and cantaloupe fruits (Liu et al., 1985), banana fruit (Inaba and Nakamura, 1986), and carnation flowers (Wang and Woodson, 1989), a large increase in ethylene production is triggered by exposure to exogenous ethylene, with activation of ACS and/or ACO. From these observations, system 2 ethylene was thought to be regulated by a positive feedback mechanism. A significant amount of ethylene is also induced by auxin or stress in a number of plant tissues, and in many cases it has been shown to be under negative feedback regulation (Yang and Hoffman, 1984). Therefore, since there are two types of large ethylene production

regulated in opposite feedback directions, the term system 2 ethylene should be limited to the ethylene produced from ripening fruits.

In tomato fruit a large body of evidence demonstrates that massive ethylene production is responsible for increases in *LE-ACS2*, *LE-ACS4*, and *LE-ACO1* transcripts (Barry et al., 1996; Lincoln et al., 1993; Olson et al., 1991; Rottmann et al., 1991; Van Der Straeten et al., 1990; Yip et al., 1992). Expression of these genes in preclimacteric tomato fruit is rapidly induced and/or enhanced by treatment with ethylene (Lincoln et al., 1993; Maunders et al., 1987; Rottmann et al., 1991). Therefore, the expression of the genes related to system 2 ethylene may be under a positive feedback regulation mechanism in tomato fruit, at least at the initiation of ripening. In section 2.1., we demonstrated the involvement of a strong positive feedback regulation mechanism in tomato fruit even at the stage of a burst in ethylene production. The increases in the abundance of *LE-ACS2*, *LE-ACS4*, and *LE-ACO1* mRNAs in ripening fruit were prevented to a large extent by treatment with MCP, an inhibitor of ethylene action. However, ethylene production, ACC content, and the activities of ACS and ACO in the fruit were not inhibited to the expected level with respect to suppression of the expression of the *ACS* and *ACO* genes, suggesting an involvement of a negatively regulated gene(s) in ethylene biosynthesis in tomato fruit.

The involvement of positive feedback regulation in ethylene biosynthesis has been elucidated at the molecular level for ACS and/or ACO in plants such as carnation (Jones and Woodson, 1997), orchid (O'Neill et al., 1993), and petunia (Tang and Woodson, 1996) flowers and mung bean (Kim and Yang, 1994) and pea (Peck and Kende, 1995) seedlings. The negative feedback regulation of ethylene biosynthesis at the molecular level has been reported in winter squash fruit (Nakajima et al., 1990), mung bean seedlings (Kim et al., 1997; Yoon et al., 1997), transgenic petunia flowers (Wilkinson et al., 1997), and leaves of the tomato cv *Never ripe* (Lund et al., 1998). Although it has been suggested that

different ACSs may be involved in the two systems of ethylene production (McGlasson, 1985), it has not been clarified which members of the *ACS* and/or *ACO* gene families are responsible for system 1 ethylene synthesis.

In this section, we demonstrate that the involvement of positive and negative feedback regulated and constitutively expressed *ACS* genes in tomato fruit, in which system 1 and system 2 ethylene production are regulated toward opposite directions of feedback, with differential expression of some members of the *ACS* gene family

Materials and Methods

Plant materials and treatments

Greenhouse-grown tomato (*Lycopersicon esculentum* Mill. cv Momotaro) fruit were harvested from a commercial farm at the following stages: immature green (about 2 weeks after flowering), mature green (pale-green color on fruit surface), turning (first appearance of pink color at blossom end), pink (pink color in approximately one-third of fruit surface), red (red color in approximately two-thirds of fruit surface), and full ripe (red color on entire fruit surface). Ethylene production by the fruit was measured at 22℃. Turning and pink fruits were treated with 10 to 20 nl•liter^{-1} MCP for 6 h and then ripened at 22℃. Ripening stages of MCP-treated fruit were monitored with reference to the color development of control fruit. Immature green fruit were treated with 5,000 μl•liter^{-1} propylene for 2 and 4 days at 22℃. Respiration and ethylene production rates, ACC content, and *in vivo* ACO activity were measured in the fruit treated with propylene. Mature green fruit were divided into three stages based on the basal level of ethylene production: MG1, MG2, and MG3. After the determination of ethylene production, pericarp tissues from the fruit equatorial region were frozen in liquid nitrogen and stored at –80℃ until RNA extraction.

All experiments except RNA extraction were repeated at least three times. MCP synthesis and treatment were carried out as described above (Section 2.1.).

Determination of ethylene biosynthesis and CO_2 production

Ethylene and CO_2 production from fruit were measured by enclosing samples in an airtight chamber for 1 h at 22℃, withdrawing for each determination 1ml of headspace gas from the chamber, and injecting into a gas chromatograph (model GC-4CMPF, Shimadzu, Kyoto, Japan) fitted with a flame-ionization detector and an activated alumina column for ethylene and into another gas chromatograph (model GC-3BT, Shimadzu) fitted with a thermal conductivity detector and a Porapack Q column for CO_2. For immature and mature green fruits, the basal level of ethylene production was measured using the mercuric perchloride method described by Akamine and Goo (1978). ACC content was measured by the method of Lizada and Yang (1979), with 80% ethanol extracts from pericarp tissues. *In vivo* ACO activity was assayed by the method of Moya-Leon and John (1994), with minor modifications. Enzyme activity was expressed as the amount of ethylene (nmol) produced per gram per hour.

RNA Extraction and RT-PCR

RNA was extracted by the hot borate method (Wan and Wilkins, 1994). Poly $(A)^+$ RNA was isolated using Oligotex-dT 30 (Takara, Kyoto, Japan) according to the manufacture's protocol. The first-strand cDNAs synthesized by the poly $(A)^+$ RNA isolated from ripe tomato fruit with or without MCP. In addition to the section 2.1., using degenerated primers, we cloned two cDNAs for *ACS* genes (*LE-ACS1A* and *LE-ACS6*), one for *ACO* gene (*LE-ACO4*), and two for ethylene receptors (Table 2.2.1.). Primers for the ethylene receptor were designed with reference to the nucleotide sequences of *eTAE1* (accession no. U41103) and *NR* (accession no. U38666), corresponding to *LeETR1* and *LeETR3* respectively

(Lashbrook et al., 1998), registered in the nucleotide sequence databases with restriction site sequences of *Bam*H Ⅰ. For amplification of the cDNA fragment of *LE-ACS3*, we used specific primers K (bp 175-201) and L (bp 822-848) designed from the given nucleotide sequences registered on the database (accession no. U17972) with restriction site sequences of *Bam*H Ⅰ and *Kpn* Ⅰ. Cycling parameters for the RT-PCR were identical to those described in section 2.1.

Amplification of full-length cDNA by RACE-PCR

To determine the full-length nucleotide sequences for *LE-ACS6* and *LE-ACO4*, RACE-PCR was performed using a cDNA amplification kit (Marathon, Clontech, Palo Alto, CA) according to the manufacture's protocol. The 5′ end fragments were amplified using specific primers N and P for *LE-ACS6* and *LE-ACO4*, respectively (Table 2.2.1.). To amplify 3′ end fragments, specific primers M and O were used for *LE-ACS6* and *LE-ACO4*, respectively (Table 2.2.1.). Each primer was designed based on the nucleotide sequences of the cDNA fragments for *LE-ACS6* and *LE-ACO4* obtained from the RT-PCR described above.

Confirmation of LE-ACS1A and LE-ACS1B expression

To determine whether *LE-ACS1A* and *LE-ACS1B* which have very high sequence similarity, were expressed in fruit tissue, a cDNA fragment was amplified on RT-PCR with a template of the combined single-strand cDNAs prepared from preclimacteric and ripening fruit in a ratio of 1:1 using specific primer pairs of G and H and I and J for *LE-ACS1A* and *LE-ACS1B*, respectively (Table 2.2.1.). These primers were synthesized with reference to the nucleotide sequences registered in the database (primer G and H, bp 958-985 and bp 1311-1334 for *LE-ACS1A* [accession no. U72389]; primers I and J, bp 958-985 and bp 1311-1337 for *LE-ACS1B* [accession no. U72390]). Competence of primers was

Tabel 2.2.1. Oligonucleotide primers used for amplification of cDNAs by RT-PCR or RACE-RCR methods.

	Name	DNAsequence	Gene
A	ACS-F	5´-cccc(ggatcc)atgggyytngcdgaraaycag-3´	degenerate
B	ACS-R	5´-cccc(ggatcc)acnarncyraarcthgacat-3´	
C	ACO-F	5´-cgc(ggatcc)gcntgysaraantggggntt-3´	
D	ACO-R	5´-aaa(ctgcag)nggytcyttngcytgraaytt-3´	
E	ETR-F	5´-gcg(ggatcc)gartgtgcwttrtggatgcca-3´	
F	ETR-R	5´-gcg(ggatcc)gctctggagttarrtcwgtttc-3´	
G	LEACS1AF	5´-gcatcaatgttgtctgatgaagtattca-3´	LE-ACS1A
H	LEACS1AR	5´-gcaatgttgttaagtccctttggc-3´	
I	LEACS1BF	5´-gcatcaatgttgtctgatgagatatttg-3´	LE-ACS1B
J	LEACS1BR	5´-gcagcaatgttgttaagtccctttgtt-3´	
K	LEACS3F	5´-gg(ggtacc)ctagcacaaaatccagacgcagctggg-3´	LE-ACS3
L	LEACS3R	5´-cg(ggatcc)gcaccaatgcgaaaaccggggagaccg-3´	
M	LEACS6RACE3	5´-gtatctcagaagtcaagagtgaagttgttgg-3´	LE-ACS6
N	LEACS6RACE5	5´-gcatccaacaacttcactcttgacttctgag-3´	
O	LEACO4RACE3	5´-cactgaagctagagaaactagctgaaaatc-3´	LE-ACO4
P	LEACO4RACE5	5´-ggatacttcaatttgatgtcctcttctgtc-3´	

confirmed by PCR with a template of genomic DNA extracted from tomato leaves. The PCR products were ligated into a plasmid, introduced into *E. coli*, and sequenced as described in section 2.1. The resulting plasmids inserted with the fragments of *LE-ACS1A* or *LE-ACS1B* were used as a template to ascertain the specificity of each primer pair in PCR. Reactions were subjected to 25 cycles of 94℃ for 1 min, 63℃ for 2 min, and 72℃ for 3 min.

The method of cloning, DNA sequencing, RNA blotting and hybridization were identical to those described in section 2.1.

Results

Isolation and identification of cDNA clones

With the cDNAs cloned in section 2.1., using degenerate and specific oligonucleotide primers (Table 2.2.1.), we cloned nine fragments from ripe tomato fruit without or treated with MCP, including five different cDNAs for *ACS* (*LE-ACS1A, LE-ACS2, LE-ACS3, LE-ACS4*, and *LE-ACS6*), two for *ACO* (*LE-ACO1* and *LE-ACO4*), and two for the ethylene receptor (*LeETR1* and *LeETR3*). Nucleotide sequences of each fragment except *LE-ACO4* were more than 99.6% identical to those of corresponding cDNA previously registered in the databases: *LE-ACS1A; LE-ACS2* (accession no. X59145); *LE-ACS3* (accession no. U17972); *LE-ACS4* (accession no. X59146); *LE-ACS6* (accession no. U74461); *LE-ACO1* (accession no. X58273); *eTAE1* and *NR*, corresponding to *LeETR1* and *LeETR3*. We have maintained the use of the term *NR* and substituted the term *LeETR1* for *eTAE1*. The mismatch of sequences between fragments and the registered cDNAs were probably due to PCR errors or differences in tomato cultivars. One fragment for *ACO* cloned in this study had low sequence similarity compared with other genes encoding ACO already

known in tomato (Barry et al., 1996), with 76% to 77% and 80% to 84% at the nucleotide and deduced amino acid levels, respectively (Table 2.2.2.). Therefore, we considered this fragment as a new member of the *ACO* gene family in tomato and registered it in the database as *LE-ACO4* (accession no. AB013101).

The full-length cDNA of *LE-ACO4*, which was obtained by RACE-PCR, contained an open reading frame of 960 bp encoding a sequence of 320 amino acids. The amino acid sequence comparison among the four tomato ACO proteins is shown in Figure 2.2.1. The *LE-ACS6* fragment cloned in this study had a completely identical sequence to an already registered *ACS* gene (Oetiker et al., 1997; accession no. U74461) except for the degenerate primer regions. The registered sequence length is limited to 308 bp and we determined full-length sequences of its cDNA using the RACE-PCR method. The full-length cDNA of *LE-ACS6* contained an open reading frame of 1431 bp encoding a sequence of 477 amino acids.

Ethylene production during fruit development and ripening and effect of MCP

Figure 2.2.2. shows the rate of ethylene production by the fruit immediately after harvest at the indicated stages and by the fruit treated with MCP at the turning or pink stages. In the control fruit ethylene production was very low at the basal level at the preclimacteric stage and increased during ripening, reaching a peak at red stage and declining slightly thereafter. This increase in ethylene production was inhibited by about 66% and 75% 2 days after MCP treatment at the turning and pink stages, respectively. Thereafter, ethylene production recovered slowly without any decline to the basal level, contrary to the expectation of the MCP action (Sisler and Serek, 1997).

Confirmation of LE-ACS1A expression in fruit tissue

Since the twin *LE-ACS1* cDNAs *LE-ACS1A* and *LE-ACS1B*, which share very

Table 2.2.2. Percentage sequence identify between ACO encoded by multigene families in tomato plant.

Deduced Amino Acid Sequence	Nucleotide Sequence			
	LE-ACO1	LE-ACO2	LE-ACO3	LE-ACO4
LE-ACO1	-	84.6	95.9	82.8
LE-ACO2	84.6	-	85.9	80.0
LE-ACO3	92.7	82.1	-	83.6
LE-ACO4	77.5	76.2	77.5	-

Figure 2.2.1. Comparsion of the deduced amino acid sequences among the four tomato ACO proteins (*LE-ACO1*; *LE-ACO2*, accession no. Y00478; *LE-ACO3*, accession no. Z54199; *LE-ACO4*). The asterisks indicate sequence identify. Highly conserved regions for ACO are boxed, and the nine shaded amino acid residues are conserved in all members of Fe (Ⅱ) ascorbate family of dioxygenases (Lasserre et al., 1996).

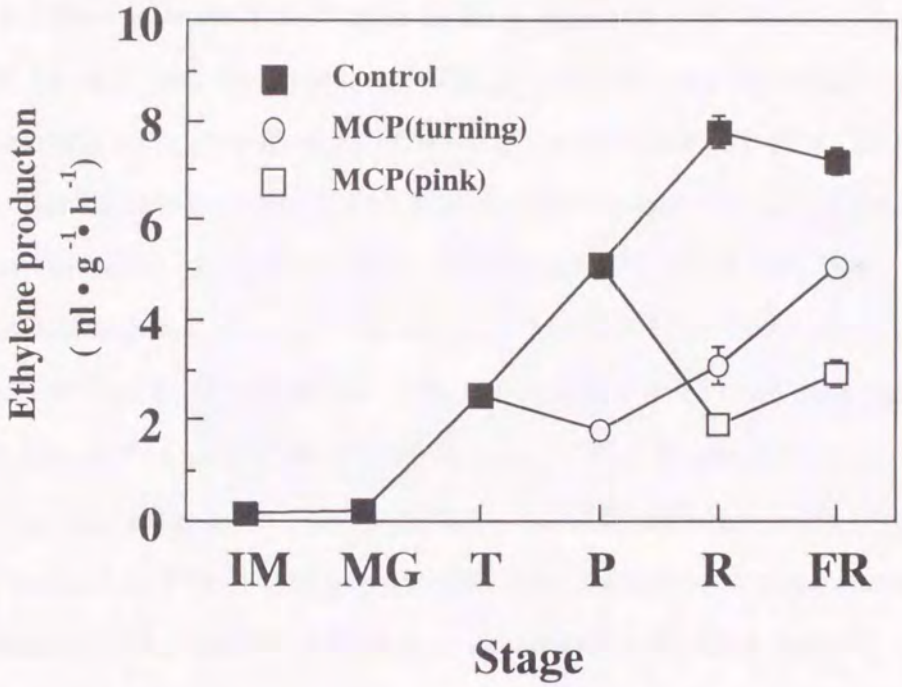


Figure 2.2.2. Changes in the rate of ethylene production in tomato fruit during development and ripening, and the effect of MCP. Fruit were harvested at six stages: immature green (IM), mature green (MG), turning (T), pink (P), red (R), and full-ripe (FR), based on the observations described in "Materials and Methods". Fruit harvested at turning and pink stages were treated with 10 to 20 nl•liter^{-1} MCP for 6 h and then ripened at 22℃. The ripening stages of MCP-treated fruit corresponding to the control fruit were determined as described in "Materials and Methods". Vertical bars are the SE of three replications; missing error bars are smaller than the symbols.

high sequence similarity, have been cloned from a tomato genomic library (Oetiker et al., 1997), we determined whether both were expressed in the fruit. As shown in Figure 2.2.3., only the *LE-ACS1A* cDNA fragment with the expected length of 377 bp was amplified by RT-PCR when the specific primers designed to have a 2-base mismatch at 3′ ends in both upstream and downstream primers (compare lanes 1 and 5) were used. The *LE-ACS1A* and *LE-ACS1B* genomic DNA fragments were amplified by PCR using each primer pair (Fig. 2.2.3., lanes 2 and 6), ligated into a plasmid, and then introduced into *E. coli*. The nucleotide sequences of each fragment were completely identical to those of the corresponding regions for each cDNA (data not shown). When these plasmids inserted with the *LE-ACS1A* or *LE-ACS1B* fragments were used as templates for PCR, the *LE-ACS1A* primer amplified the *LE-ACS1A* fragment but not the *LE-ACS1B* fragment (Fig. 2.2.3., compare lanes 3 and 8) and vice versa (Fig. 2.2.3., compare lanes 4 and 7). These experiments confirmed that, among the twin *LE-ACS1* genes, only *LE-ACS1A* mRNA was expressed in the fruit tissue.

Gene expression during fruit development and ripening and effect of MCP

Figure 2.2.4. shows the expression of members of the gene families for *ACS*, *ACO*, and ethylene receptor in tomato fruit during development and ripening and in the fruit treated with MCP. Among the five members of the *LE-ACS* gene family, the abundance of *LE-ACS2* and *LE-ACS4* mRNAs in the fruit was undetectable in fruit at the preclimacteric stage, increased from the turning to pink stages, and thereafter slightly declined (Fig. 2.2.4., lanes 1-6). These increases in the mRNA abundance associated with ripening were prevented to a large extent by treatment of fruit with MCP at both the turning (Fig. 2.2.4., lanes 7-9) and pink (Fig. 2.2.4., lanes 10 and 11) stages. In particular, 2 days after MCP treatment, the abundance of mRNA that hybridized with the *LE-ACS2* and *LE-ACS4* probes was almost completely eliminated (Fig. 2.2.4., compare lanes 4

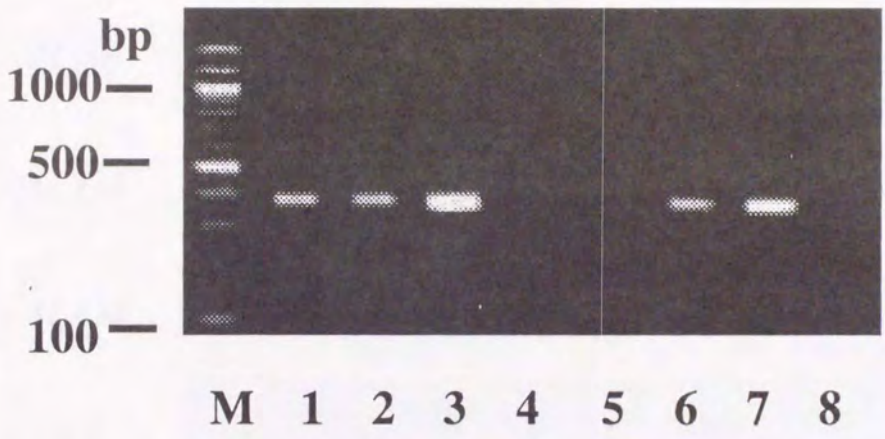


Figure 2.2.3. Agarose/ethidium bromide gel image of RT-PCR products amplified using specific primers for *LE-ACS1A* and *LE-ACS1B*. Each primer was designed to amplify the corresponding region in *LE-ACS1A* and *LE-ACS1B* but with two differnt nucleotides at the 3' ends either upstream or downstream set to avoid cross-amplification. The *LE-ACS1A* primers were used for the reaction of lanes 1, 2, 3, and 8, and the *LE-ACS1B* primers were used for lanes 4 to 7. Templetes used for RT-PCR were the combined single-strand cDNAs prepared from preclimacteric and ripening fruits in a ratio of 1:1 (lanes 1 and 5), the genomic DNA extracted from tomato leaves (lanes 2 and 6), and the plasmid inserted with the *LE-ACS1A* (lanes 3 and 4) or *LE-ACS1B* (lanes 7 and 8) fragment. Lane M shows a 100-bp DNA ladder as a size marker.

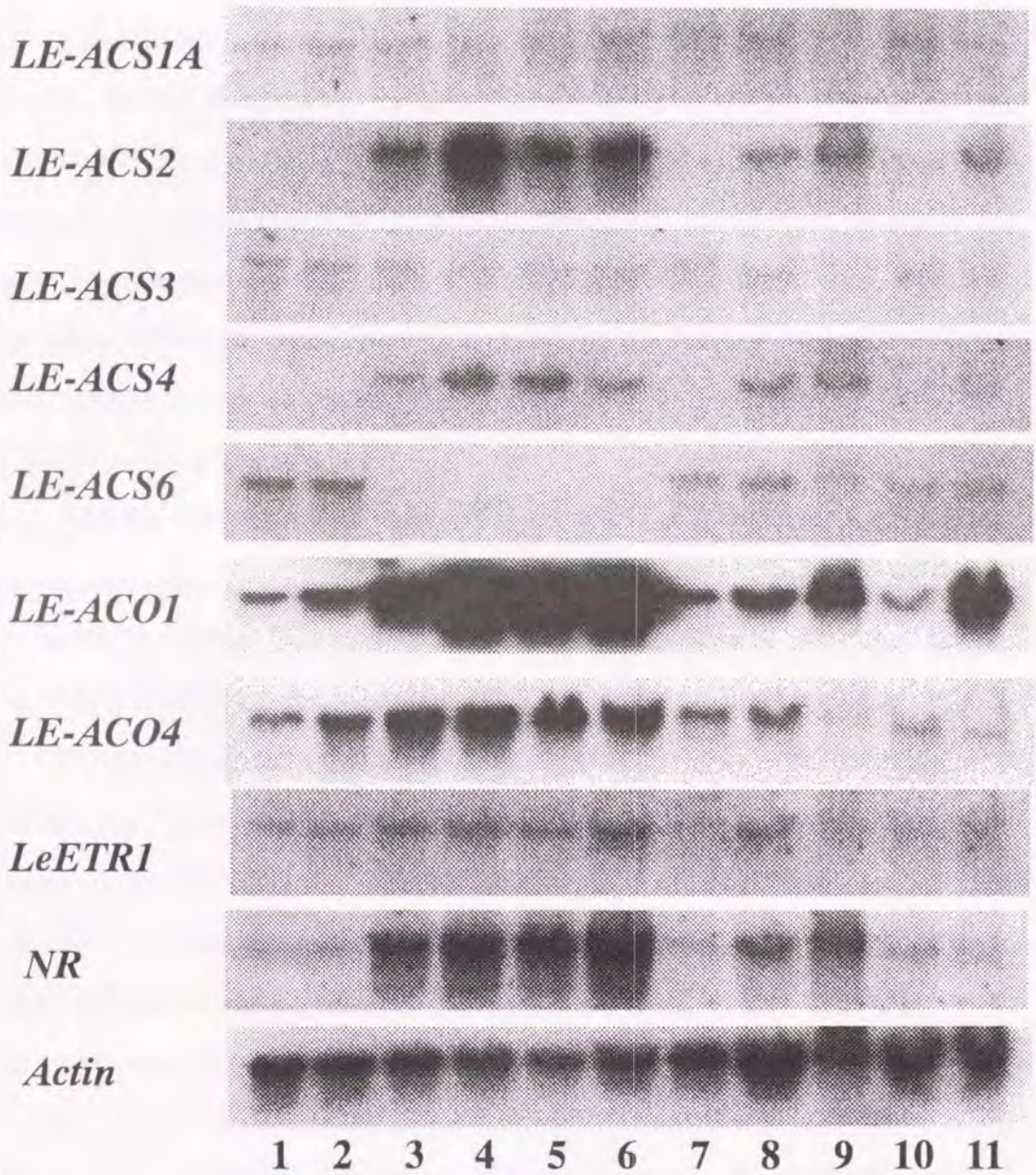


Figure 2.2.4. Expression of *LE-ACS*, *LE-ACO*, and ethylene receptor genes in tomato fruit during development and ripening and effect of MCP. mRNAs were prepared from the fruit immediately after the determination of ethylene levels as shown in Figure 2.2.2. Lane 1, control fruit at the immature stage; lane 2, control fruit at the mature green stage; lane 3, control fruit at the turning stage; lane 4, control fruit at the pink stage; lane 5, control fruit at the red stage; lane 6, control fruit at the full- ripe stage; lane 7, turning-stage fruit 2 days after MCP treatment; lane 8, turning-stage fruit 4 days after MCP treatment; lane 9, turning-stage fruit 6 days after MCP treatment; lane 10, pink-stage fruit 2 days after MCP treatment; and lane 11, pink-stage fruit 4 days after MCP treatment. Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amount of mRNA loaded.

and 5 with 7 and 10, respectively). This elimination recovered gradually in 2 and 4 days (lanes 8, 9, and 11).

In contrast, the *LE-ACS6* gene was expressed in the fruit at the immature green and mature green stages (Fig. 2.2.4., lanes 1 and 2), whereas no signal for this gene was detected in the ripening fruit (Fig. 2.2.4., lanes 3-6). However, accumulation of *LE-ACS6* mRNA was detected in the fruit treated with MCP at both the turning and pink stages (Fig. 2.2.4., lanes 7-11). *LE-ACS1A* and *LE-ACS3* genes were expressed weakly in the fruit throughout development and ripening, and the abundance of their mRNAs was less influenced by treatment with MCP. Although two *LE-ACO* genes were expressed in immature green and mature green fruit (Fig. 2.2.4., lanes 1 and 2), the abundance increased further upon commencement of ripening (Fig. 2.2.4., lanes 3-6), particularly in *LE-ACO1*. The increases in accumulation of the *LE-ACO* mRNAs with ripening were prevented considerably by treatment of fruit with MCP at both the turning and pink stages (Fig. 2.2.4., lanes 7-11). Of the two members of the ethylene receptor gene family, the abundance of *NR* mRNA in the fruit was at a very low level at the preclimacteric stage (Fig. 2.2.4., lanes 1 and 2), increased suddenly at the turning stage, and maintained its strong signals during ripening (Fig. 2.2.4., lanes 3-6). This increase of *NR* mRNA associated with ripening was also lowered by MCP treatment in a manner similar to that observed for *LE-ACS2* (Fig. 2.2.4., lanes 7-11). Signals for the *LeETR1* gene in the fruit were detected at the preclimacteric stage (Fig. 2.2.4., lanes 1 and 2) and increased slightly during ripening (Fig. 2.2.4., lanes 3-6). MCP decreased the abundance of *LeETR1* mRNA in ripening fruit (Fig. 2.2.4., lanes 7-11).

Effect of propylene on gene expression in immature green fruit

The results presented above suggest that the expression of the *LE-ACS6* gene may be under negative feedback regulation in tomato fruit. To test this

hypothesis, immature green fruit were treated with 5000 $\mu l \cdot liter^{-1}$ propylene for 2 and 4 days. Neither autocatalytic ethylene production nor increases in respiration rate and ACC content were induced by propylene in these young fruit, whereas ACO activity was activated more than 2- to 3-fold (Table 2.2.3). The results of northern analysis for mRNAs from these fruit are shown in Figure 2.2.5. The accumulation of *LE-ACS6* transcript in the control fruit (Fig. 2.2.5., lanes 1-3) was strongly prevented by treatment with propylene for 2 and 4 days (Fig. 2.2.5., lanes 4 and 5, respectively). Since there were no increases in ethylene production or ACC content in the fruit, propylene did not induce the accumulation of transcripts for *LE-ACS2* and *LE-ACS4*. *LE-ACS1A* and *LE-ACS3* were expressed constitutively in the fruit irrespective of propylene treatment. Although in vivo activity of ACO in the fruit was increased by propylene treatment, we did not observe an enhancement in the accumulation of *LE-ACO1* mRNA. Signals for the *LeETR1* and *NR* genes were weak in the control fruit and were less influenced by treatment with propylene.

Transition of expression of genes at ripening onset

It is possible that the elimination of *LE-ACS6* and the appearance of *LE-ACS2* transcripts may have been responsible for the transition from system 1 to system 2 ethylene production. To examine this concept, northern analysis was performed in fruit at stages from mature green to turning, all of which had different levels of basal ethylene production (Fig. 2.2.6.). The rates of ethylene production in the fruit were 0.18, 0.36, 0.96, and 1.46 $nl \cdot g^{-1} \cdot h^{-1}$ at the MG1, MG2, MG3, and turning stages, respectively. The abundance of *LE-ACS6* mRNA in the fruit decreased gradually with ripening, reaching undetectable levels at the turning stage. In contrast, the *LE-ACS2* transcript, which was undetectable at the MG1 stage, increased gradually when the rate of ethylene production was increased. Signals for the *NR* gene at the MG1 stage were very weak, increasing

Table 2.2.3. Effect of propylene on the rates of respiration and ethylene production, ACC content, and *in vivo* ACO activity in immature green fruit.

Treatment time (day)	Respiration (μl $CO_2 \cdot g^{-1} \cdot h^{-1}$)		Ethylene (pmol $\cdot g^{-1} \cdot h^{-1}$)		ACC (nmol $\cdot g^{-1}$)		ACO (nmol ethylene $\cdot g^{-1} \cdot h^{-1}$)	
	Control	Propylene	Control	Propylene	Control	Propylene	Control	Propylene
0	46.7±4.6		7.9±3.1		0.32±0.04		0.48±0.15	
2	20.8±1.9	23.0±4.0	8.8±2.5	6.9±2.0	0.32±0.06	0.35±0.05	0.42±0.08	1.02±0.18
4	24.1±2.8	24.4±1.4	8.8±1.3	7.0±3.0	0.32±0.05	0.40±0.03	0.55±0.09	1.67±0.35

Fruit were harvested about 2 weeks after flowering and then treated with 5,000 μl $\cdot$ liter^{-1} propylene for 2 and 4 days at 22℃. The values are the means ±SE of three replications.

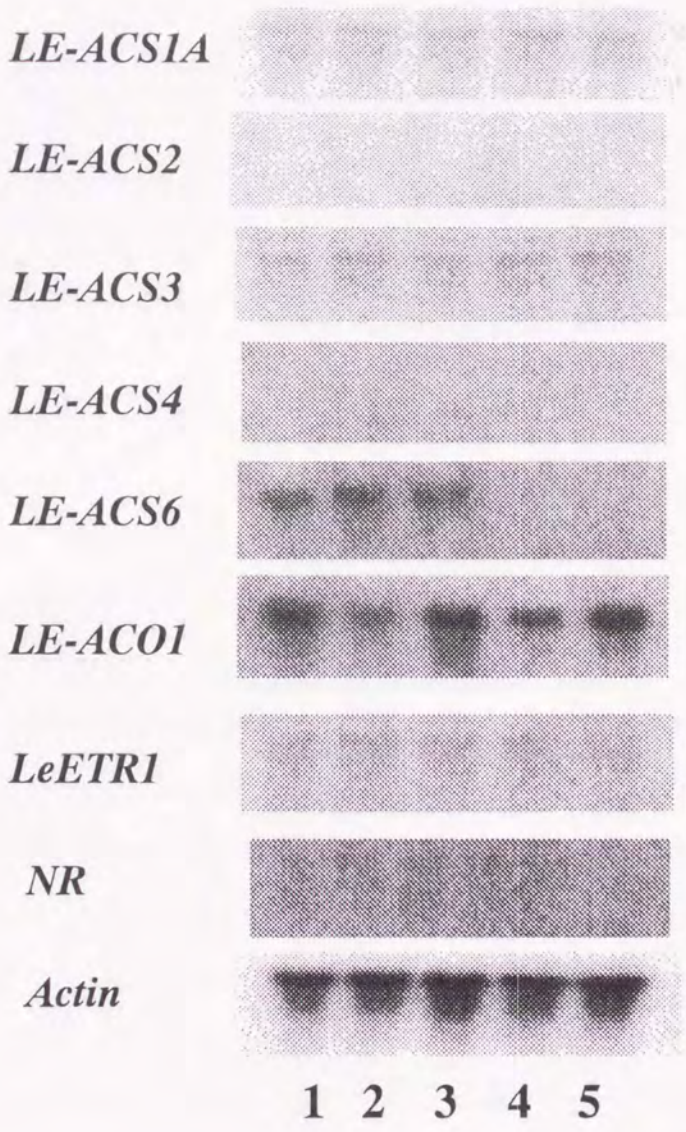


Figure 2.2.5. Effect of propylene on the accumulation of mRNAs corresponding to *LE-ACS* and ethylene receptor gene families and the *LE-ACO1* gene in immature green fruit. mRNAs were isolated from the same fruit sample shown in Table 2.2.3. Lane 1, control fruit at harvest; lane 2, control fruit 2 days after harvest; lane 3, control fruit 4 days after harvest; lane 4, propylene-treated fruit for 2 days; lane 5, propylene-treated fruit for 4 days. Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amount of mRNA loaded.

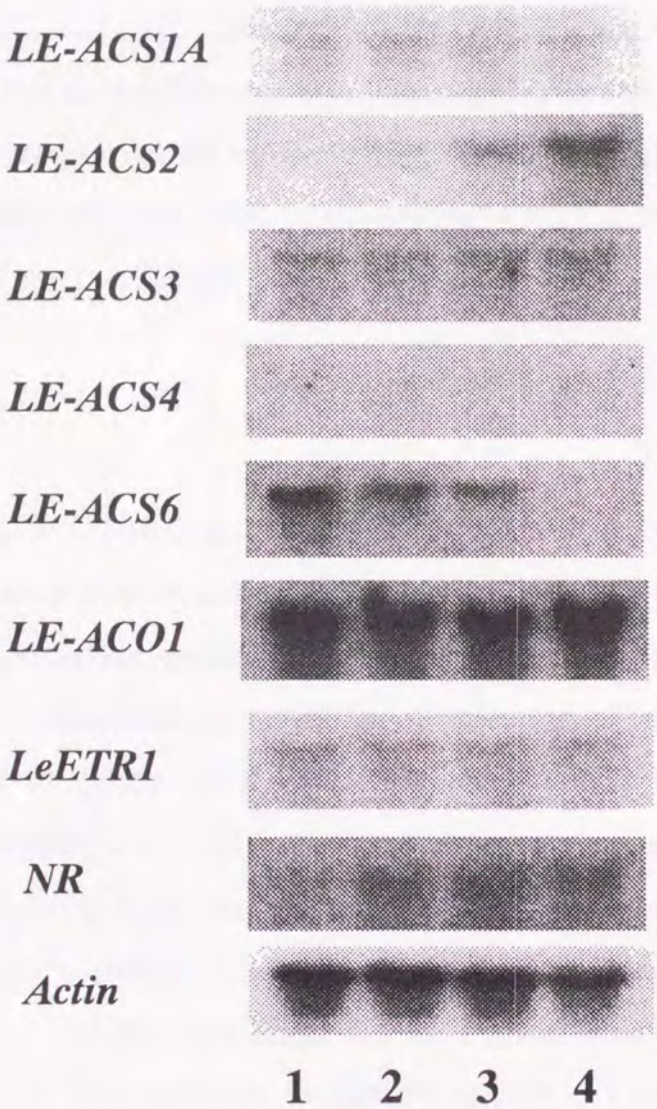


Figure 2.2.6. Changes in the accumulation of mRNAs corresponding to *LE-ACS* and ethylene receptor gene families and the *LE-ACO1* gene in fruit with different rates of ethylene production from the mature green stage to the turning stage. Lane 1, MG1 fruit (0.18 nl• g^{-1}•h^{-1} ethylene production); lane 2, MG2 fruit (0.36 nl•g^{-1}•h^{-1} ethylene production); lane 3, MG3 fruit (0.96 nl•g^{-1}•h^{-1} ethylene production); and lane 4, turning fruit (1.46 nl•g^{-1}•h^{-1} ethylene production). Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amount of mRNA loaded.

from the MG2 stage to the turning stage. Signals for the *LE-ACS1A* and *LE-ACS3* genes changed little from the MG1 stage to the turning stage. The abundance of *LE-ACO1* and *LeETR1* mRNAs was also unchanged from the MG1 stage to the turning stage. No signal for the *LE-ACS4* gene was detected in the turning fruit, which had a lower ethylene level (1.46 $nl \cdot g^{-1} \cdot h^{-1}$) than that used in the fruit shown in Figure 2.2.2. and 2.2.4. (2.35 $nl \cdot g^{-1} \cdot h^{-1}$).

Discussion

The climacteric life of fruits is divided into preclimacteric and climacteric stages depending on whether a massive production of ethylene has commenced. In tomato fruit ethylene production during the climacteric stage has been demonstrated to be due to the accumulation of transcripts of two *ACS* genes, *LE-ACS2* and *LE-ACS4* (Lincoln et al., 1993; Rottmann et al., 1991), and one *ACO* gene, *LE-ACO1* (Barry et al., 1996). Using MCP, an ethylene action inhibitor, we previously demonstrated that the expression of all three of genes is highly regulated through a positive feedback mechanism in ripening tomato fruit (Section 2.1.). In that study we suggested the possible existence of a gene(s) under negative feedback regulation, because the inhibitory effects of MCP on the expression of the genes were not correlated with those on ethylene biosynthesis. To provide experimental evidence to support our hypothesis, we cloned nine cDNA fragments, including five members of the *ACS* gene family, two of the *ACO* family, and two of the ethylene receptor family. Among the seven previously cloned genes for ACS (Lincoln et al., 1993; Oetiker et al., 1997; Olson et al., 1995; Rottmann et al., 1991; Spanu et al., 1993; Yip et al., 1992), fragments of *LE-ACS1B* and *LE-ACS5* could not be amplified by RT-PCR used in this study, even by the use of specific primers. Although the transcription of these two genes has been demonstrated in tomato roots and suspension cultures

(Oetiker et al., 1997; Spanu et al., 1993; Yip et al., 1992), there is no evidence demonstrating their expression in the fruit. Therefore, we concluded that their transcripts were absent in the fruit tissue.

In this section, we observed large ethylene production in the fruit from the turning stage with further increases toward the red stage (Fig. 2.2.2.). This increase in ethylene production was prevented to a large extent by treatment with MCP at both the turning and pink stages. Using mRNAs extracted from this fruit, we performed northern analysis with the probes prepared from cDNA fragments cloned in this study (Fig. 2.2.4.). Among five members of the *LE-ACS* gene family, the abundance of *LE-ACS2* and *LE-ACS4* mRNAs in the fruit increased beginning at the turning stage, and MCP greatly suppressed this increase in a manner similar to that observed in Section 2.1. In mature green fruit the transcripts of these genes were absent but were inducible by treatment with ethylene through a positive feedback mechanism, resulting in the induction of ripening (Lincoln et al., 1993).

Expression of *LE-ACS2* during the natural progress of ripening first appeared in MG2 fruit, the stage showing the first elevation of ethylene production from the basal level (Fig. 2.2.6.). However, propylene did not induce the accumulation of *LE-ACS2* and *LE-ACS4* transcripts in immature green fruit within 4 days (Fig. 2.2.5.) but did by 8 days of treatment (data not shown), indicating a possible lack of a rapid autocatalytic system for ethylene biosynthesis in young fruit. This lack of a rapid response to applied ethylene has been reported in young tomato fruit, in which fruits harvested as early as 17 days after pollination required 12 to 15 days of continuous treatment with 1000 $\mu l \cdot liter^{-1}$ ethylene to develop red color (Lyons and Pratt, 1964). Although expression of the *LE-ACS2* and *LE-ACS4* genes is also inducible by wounding (Lincoln et al., 1993), these are probably the major genes responsible for the system 2 ethylene production during ripening in tomato fruit. More direct evidence for this is shown in transgenic tomatoes in

which the *LE-ACS2* antisense fruits produce less ethylene and fail to ripen, with complete inhibition of the *LE-ACS2* and *LE-ACS4* genes during ripening (Oeller et al., 1991).

In contrast to *LE-ACS2* and *LE-ACS4*, the *LE-ACS6* gene was expressed in fruit from the immature green to the mature green stages, whereas no signal for this gene was detected in the ripening fruit. Signals for this gene were detected in the ripening fruit treated with MCP (Fig. 2.2.4.), strongly suggesting that the expression of the *LE-ACS6* gene is regulated by a negative feedback mechanism. This concept was clearly demonstrated in immature green fruit, in which the previously detected signals for the *LE-ACS6* gene were eliminated by treatment with propylene, an ethylene analog (Fig. 2.2.5.). Furthermore, the abundance of this mRNA in the fruit during the natural onset of ripening decreased gradually to an undetectable level at the turning stage (Fig. 2.2.6.).

Oetiker et al. (1997) isolated *LE-ACS6* cDNA from tomato roots, and this is the only available information concerning its expression, which suggested that it exhibits an elicitor–inducible feature. Lincoln et al. (1993) also previously described the cloning of *LE-ACS6* cDNA and suggested the possible expression of this gene in ripe tomato fruit. However, their suggestion differs from our present observation with respect to the characteristic features of the *LE-ACS6* gene. Therefore, *LE-ACS6* reported by Lincoln et al. (1993) may have been a different cDNA from that cloned by Oetiker et al. (1997) and that obtained in the present study. Mori (1995) described an expression pattern of *LE-ACS6* in tomato fruit that is similar to ours, with and elimination of its transcripts in ripe fruit, but to our knowledge, no further information is available for this observation (in particular the gene sequences). The present results clearly demonstrate the existence of an ethylene-biosynthetic gene, the expression of which is regulated under a negative feedback mechanism in fruit. The possible involvement of a negative feedback regulation at the ethylene-production level

has been suggested in fruits such as banana (Vendrell and McGlasson, 1971), *Citrus* (Riov and Yang, 1982), and winter squash (Hyodo et al., 1985).

LE-ACS1A and *LE-ACS3* genes were expressed in the fruit throughout development and ripening (Fig. 2.2.4. and 2.2.6.). Furthermore, the abundance of their mRNAs was not influenced by treatment with either MCP (Fig. 2.2.4.) or propylene (Fig. 2.2.5.), indicating that the expression of these genes is independent of ethylene action. Although these two genes resembled each other closely in expression pattern, *LE-ACS3* had low sequence similarities (less than 62%) among the *LE-ACS* gene family (data not shown). This may exclude a possibility that the probe for *LE-ACS3* could hybridize to other transcripts encoding tomato ACS. The full-length sequence of *LE-ACS1A* mRNA together with its twin of *LE-ACS1B* was previously registered on the database (accession no. U72389 and U72390), and their expression was first examined in cultured cells using the RNase-protection assay, in which *LE-ACS1B* was strongly and constitutively expressed but no signal for *LE-ACS1A* was detectable (Oetiker et al., 1997). However, only the *LE-ACS1A* cDNA fragment was amplified on RT-PCR. *LE-ACS5* was not amplified in the present study, suggesting a tissue-specific expression of each *ACS* gene family. The transcript of *LE-ACS3* has been detected in fruits (Yip et al., 1992) and suspension cultures (Oetiker et al., 1997). Among the members of the *LE-ACS* gene family studied, *LE-ACS1A*, *LE-ACS3*, and *LE-ACS6* genes were expressed in the preclimacteric fruit, suggesting that system 1 ethylene in tomato fruit may be mediated via these three genes.

In tomato at least three genes encode ACO (Barry et al., 1996): *LE-ACO1* is the main gene expressed in ripening tomato fruit, *LE-ACO2* expression is mainly restricted to the tissues associated with the anther cone, and *LE-ACO3* transcripts accumulate in floral organs and transiently appear with a weak signal in fruit at the breaker stage (Barry et al., 1996). In the present study we cloned a novel *ACO* gene and named it *LE-ACO4*. Both *LE-ACO1* and *LE-ACO4* transcripts

accumulated in preclimacteric fruit, and this accumulation increased in ripening fruit. This increase was prevented to a large extent by MCP treatment in a manner similar to that of the *LE-ACS2* and *LE-ACS4* genes (Fig. 2.2.4.).

Although feedback regulation of the *ACO* genes has not yet been clarified, there is evidence that accumulation of the transcripts is enhanced with increases in ethylene production and by exogenously applied ethylene in fruits such as tomato (Barry et al., 1996), apple (Ross et al., 1992), melon (Lasserre et al., 1996), banana (Huang et al., 1997), kiwifruit (Whittaker et al., 1997), and pear (Lelievre et al., 1997). In vegetative tissues *ACO* mRNA has also been shown to be regulated by ethylene; the transcript for an *ACO* gene in excised mung bean hypocotyls was enhanced by exogenous ethylene and suppressed by aminooxyacetic acid, an ACS inhibitor, with a reduction of endogenous ethylene to the basal level (Kim and Yang, 1994). From these observations, it may be reasonable to assume that a positive feedback regulation is involved in the expression of *ACO* gene in a manner similar to that in *ACS*. However, since propylene did not enhance the already-accumulated *LE-ACO1* transcript in immature green fruit (Fig. 2.2.5.), the responsiveness of *LE-ACO1* to ethylene may be less than that of *LE-ACS6*.

Since the *ETR1* gene in *Arabidopsis* was cloned and sequenced as the gene related to ethylene receptors (Chang et al., 1993), five homologs (*Le-ETR* 1-5) have been isolated from tomato (Lashbrook et al., 1998). We cloned cDNA fragments corresponding to the *Le-ETR1* and *Le-ETR3*, which were first cloned by Zhou et al. (1996) and Wilkinson et al. (1995), genes based on their reported sequences. Expression of the *NR* gene was extremely low in immature and mature green fruit but suddenly increased greatly at the turning stage (Fig. 2.2.4.). Investigations at the onset of ripening revealed that this increase commenced in MG2 fruit, the stage of the first increase in ethylene production from the basal level (Fig. 2.2.6.). Wilkinson et al. (1995) indicated that *NR*

mRNA in tomato fruit is positively regulated by ethylene in a development-specific manner from observations that the amount of *NR* mRNA increases in ripening fruit and ethylene-treated mature green fruit but not in *Nr* mutant tomato.

A strong induction of *NR* mRNA at the onset of ripening has also been demonstrated in tomato fruit (Lashbrook et al., 1998). In the present study this accumulation of *NR* mRNA associated with ripening was prevented in the fruit treated with MCP (Fig. 2.2.4.). It has been proved that MCP is an ethylene-action inhibitor that binds to the receptor site competitively, thereby preventing tissue response to ethylene (Sisler and Serek, 1997). The present results demonstrate that MCP prevents the accumulation of *LE-ACS2*, *LE-ACS4*, *LE-ACO1* and *LE-ACO4* mRNAs in the ripening fruit with an almost complete elimination of *NR* transcripts (Fig. 2.2.4.). Furthermore, inhibition of the accumulation of *LE-ACS* and *LE-ACO* transcripts recovered after 2 to 4 days concomitantly with the recovery of *NR* transcripts. A similar observation has been reported for tomato fruit using diazocyclopentadiene, another inhibitor of ethylene action (Tian et al., 1997).

The above observations, together with the results presented here, suggest that the NR protein may be synthesized successively in tomato fruit during ripening, leading to the recovery of the gene transcripts that are regulated under positive feedback. The present results also suggest that this successive synthesis of NR protein might be under the control of a positive feedback mechanism. However, the expression of this gene was not inducible in immature green fruit by exposure to ethylene for 1 day (Wilkinson et al., 1995) or propylene for 4 days (Fig. 2.2.5.). These differences in *NR* gene expression in response to ethylene treatment between immature and ripening fruits may modulate the differential sensitivity to ethylene in maturing tomato fruits (Wilkinson et al., 1995). McGlasson (1985) previously pointed out that most fruit become increasingly

sensitive to ethylene with time after anthesis. The abundance of *LeETR1* mRNA accumulated constitutively throughout development and ripening irrespective of treatment with either MCP or propylene. Similar results have been reported for tomato leaf, flower, and fruit tissues, in which expression was unaffected by ethylene, silver ions, an ethylene-action inhibitor, or auxin in leaf-abscission zones (Zhou et al., 1996). Using the RNase-protection assay, Lashbrook et al. (1998) recently demonstrated that the signals for three members of *ETR1* homologs, including *LeETR1* and *NR*, were detectable in tomato fruit throughout preclimacteric stages. Therefore, the presence of one or more *ETR1* homologs prior to ripening may contribute to ripening-independent ethylene perception processes in immature fruit, by which propylene eliminated the *LE-ACS6* transcript but did not induce the *LE-ACS2* transcript (Fig. 2.2.5.).

In conclusion, the results presented here suggest that ethylene biosynthesis in tomato fruit is regulated by the three different groups of the *ACS* gene family: (a) *LE-ACS2* and *LE-ACS4* are the dominant genes responsible for system 2 ethylene production in ripening fruit and their expression is regulated by a positive feedback mechanism, (b) the *LE-ACS6* gene is responsible for the low rates of system 1 ethylene production and is negatively regulated in preclimacteric fruit, and (c) the *LE-ACS1A* and *LE-ACS3* genes are also responsible for the preclimacteric system 1 ethylene production, and their transcripts accumulate constitutively throughout fruit development irrespective of the mode of feedback regulation.

In tomato fruit, the preclimacteric system 1 ethylene production is mediated by the *LE-ACS1A*, *LE-ACS3*, and *LE-ACS6* genes, together with *LE-ACO1* and *LE-ACO4*. Ethylene production shifts to system 2 at the climacteric stage, with a burst in the accumulation of *LE-ACS2*, *LE-ACS4*, *LE-ACO1*, and *LE-ACO4* mRNAs as a result of positive feedback regulation. This transition from system 1 to system 2 ethylene production may be controlled by the accumulated level of

NR protein from the mature green stage to the turning stage.

Summary

We investigated the feedback regulation of ethylene biosynthesis in tomato (*Lycopersicon esculentum*) fruit with respect to the transition from system 1 to system 2 ethylene production. The abundance of *LE-ACS2*, *LE-ACS4*, and *NR* mRNAs increased in the ripening fruit concomitant with a burst in ethylene production. These increases in mRNAs with ripening were prevented to a large extent by treatment with MCP, an ethylene action inhibitor. Transcripts for the *LE-ACS6* gene, which accumulated in preclimacteric fruit but not in untreated ripening fruit, did accumulate in ripening fruit treated with MCP. Treatment of young fruit with propylene eliminated the accumulation of transcripts for this gene. *LE-ACS1A*, *LE-ACS3*, and *LeETR1* genes were expressed constitutively in the fruit throughout development and ripening irrespective of whether the fruit was treated with MCP or propylene. The transcripts for *LE-ACO1* and *LE-ACO4* genes already existed in preclimacteric fruit and increased greatly when ripening commenced. These increases in *LE-ACO* mRNA with ripening were also prevented by treatment with MCP. The results suggest that in tomato fruit the preclimacteric system 1 ethylene is possibly mediated via constitutively expressed *LE-ACS1A* and *LE-ACS3* and negatively feedback-regulated *LE-ACS6* genes with preexisting *LE-ACO1* and *LE-ACO4* mRNAs. At the onset of the climacteric stage, it shifts to system 2 ethylene, with a large accumulation of *LE-ACS2*, *LE-ACS4*, *LE-ACO1*, and *LE-ACO4* mRNAs as a result of a positive feedback regulation. This transition from system 1 to system 2 ethylene production might be related to the accumulated level of *NR* mRNA.

Chapter 3. Identification of ethylene-responsive elements in promoter region of two ACC synthase genes regulated in opposite feedback directions

Introduction

In the previous Chapter, we demonstrated that ethylene biosynthesis in tomato fruit is regulated by the three different groups of the *ACS* gene family: (a) the expressions of *LE-ACS2* and *LE-ACS4* are regulated by a positive feedback mechanism in ripening fruit (b) *LE-ACS6* is negatively regulated in preclimacteric fruit, and (c) transcripts of *LE-ACS1A* and *LE-ACS3* accumulate constitutively throughout fruit development irrespective of the mode of feedback regulation. From these results, we had strong interest in two different *ACS* genes, *LE-ACS2* and *LE-ACS6*, that were regulated in opposite directions of feedback mechanism.

Sequences of genomic DNA encoding ACS have been determined from several plant species such as zucchini (Huang et al., 1991), tomato (Lincoln et al., 1993; Olson et al., 1995; Rottmann et al., 1991; Shiu et al., 1998), *Arabidopsis* (Abel et al., 1995; Liang et al., 1992, 1993, 1995, 1996; Van Der Straeten et al., 1992), rice (Zarembinski and Theologis, 1993), potato (Destefano-Beltran et al., 1995), winter squash (accession no. U37774 Nakagawa et al., 1996), mung bean (Yoon et al., 1999), petunia (Lindstrom et al., 1999), and apple (Sunako et al., 1999), and their structures have been characterized. However, analysis of promoter activity using the 5′-flanking region has been performed for *ACS1* (previously referred as *AT-ACC1* by Van Der Straeten et al., 1992 or *ACC2* by Liang et al., 1992, respectively) in transgenic *Arabidopsis* (Rodrigues-Pousada et al., 1993), *VR-ACS6* in transgenic tobacco (Yoon et al., 1999) and *PH-ACS2* in transgenic petunia pollen (Lindstrom et al., 1999). On the other hand, genomic

sequences of *ACO* have been determined in *petunia hybrida* (Tang et al., 1993), melon (Lasserre et al., 1996,1997), tomato (Blume et al., 1997a), banana (Huang et al., 1997; Lopez-Gomez et al., 1997) and apple fruit (Atkinson et al., 1998). These promoter activities were analyzed for *CM-ACO1* and *CM-ACO3* in transgenic tobacco (Lasserre et al., 1997), for *LE-ACO1* in transgenic tomato and *Nicotiana plumbaginifolia* (Blume and Grierson, 1997b) and for an apple *ACO* gene (accession no AF030859) in transgenic tomato (Atkinson et al., 1998). However, there is no much evidence about the role of *ACS* and *ACO* promoters in the positive or negative feedback regulation of ethylene in fruit tissue, except for *LE-ACO1* which is induced 2.5 to 5 fold after exposing mature green tomato fruit to ethylene.

In this Chapter, 5´-flanking regions of *LE-ACS2* and *LE-ACS6* genes in tomato fruit were analyzed to elucidate a possible mechanism of positive and negative feedback regulation of ethylene biosynthesis.

Materials and Methods

Plant materials

For the cloning of *LE-ACS2* and *LE-ACS6* promoter regions, tomato (*Lycopersicon esculentum* Mill. cv Momotaro) genomic DNA was extracted from young expanding leaves by the method of Murry and Thompson (1980). Tomato fruits were harvested at immature green and pink stages to compare the promoter activity in the fruit at the unripe and ripe stages using ß-glucuronidase (GUS) transient assay. Pericarp slices for the GUS transient assay were cut from equatorial region of the fruit. To avoid the action of wound-ethylene which may be produced upon slicing for the control immature green fruit, were treated with MCP. In order to test the effect of ethylene on promoter activity, immature green and pink stage fruit were treated with propylene and MCP, respectively. The

treatments were carried out in the same way described in the former Chapter.

Cloning the sequence of 5′ flanking region of LE-ACS6

From the coding region of *LE-ACS6* (accession no. AB013100), gene specific primers were synthesized and genomic DNA fragments were amplified using inverse PCR (IPCR) according to the method described by Ochman et al. (1988) and Triglia et al. (1988). The conditions for the IPCR were as follows; 1 min 94℃, 2 min 55℃, 3 min + 10 sec extension/cycle 72℃ for 30 cycles. The amplified genomic DNA fragments were cloned into pGEM-T vector (Promega) and sequenced using DNA sequencers (DSQ-1000L, Shimadzu). The sequences of genomic DNA were determined by overlapping the obtained sequence with the known regions.

Plasmid construction of ACS promoter-GUS reporter gene

From the already determined sequence of 5′-flanking for *LE-ACS2* (accession no. X59145), specific primers A and B were synthesized with restriction site sequences of *Pst* Ⅰ and *Bam* HⅠ (Table 3.1.). Primers C and D for *LE-ACS6* were synthesized from the obtained nucleotide sequences in this Chapter. These primers were similar to the ones referred to in Figure 3.1., with restriction site sequences of *Xba* Ⅰ and *Sma* Ⅰ/*Kpn* Ⅰ. Objective promoter regions of about 2.4 and 2.2 kb for *LE-ACS2* and *LE-ACS6*, respectively, were amplified by PCR with genomic DNA from tomato as a template. The restriction site sequences were selected as optimal restriction enzymes that facilitate the subcloning of the PCR products into the pBI221 plasmid (CLONTECH) which contains cauliflower mosaic virus (CaMV) 35S promoter, GUS and nopaline synthase (NOS) terminator. The plasmid was cut with the respective restriction enzyme, and the PCR products were subcloned into the plasmid by replacing CaMV 35S promoter to construct the *ACS* promoter-GUS chimeric gene. A plasmid without

Table 3.1. Oligonucleotide primers used for amplification of genomic DNA by PCR.

Name	DNA sequence	gene
A ACS2-F	5´-cc(aagctt)gatgatcaaatctactttgaagtcca-3´	LE-ACS2
B ACS2-R	5´-cgc(ggatcc)aagagaattaaggaatgtgaggg-3´	
C ACS6-F	5´-gc(tctaga)gcgataattgtcattctcgtatatgtc-3´	LE-ACS6
D ACS6-R	5´-gg(ggtacccggg)tggctaatttgctaatatgtagacc-3´	

CaMV 35S promoter prepared by deleting CaMV 35S promoter from pBI221 (pBI-35S) and original pBI221 plasmid were also used as negative and positive control constructs, respectively. The construct of the resulting plasmids and the deletion endpoindts were confirmed by DNA sequence analysis.

Delivery of particles by bombardment

After construction, the above described plasmid DNAs were transformed into *E. coli* (JM109, Takara, Japan) and cultured in LB medium, purified with QIAprep Spin Miniprep Kit (QIAGEN) and coated onto tungsten particles according to Takeuchi et al. (1992) by ethanol precipitation. The DNA-coated particles were (DNA 25ng + tungsten 12.5 $\mu g \cdot \mu l^{-1}$100% Et-OH) delivered with a particle bombardment device (IDERA GIE-III type, TANAKA, Hokkaido, Japan) into tissue slices (diameter 1.5 cm, thickness 1.3 and 2.6 mm for immature and pink stages tomato, respectively) prepared with corkborer and laser blade from the pericarp tissue. The conditions for bombardment were as follows; accelerating pressure was 5 kg cm^{-2}, stopper-to- target-cell distance was 3 cm and partial vacuum in sample chamber was about 680 mmHg. The bombarded tissues were placed in sterile petridishes and incubated at 25℃ for 24 h under humidified conditions.

GUS transient assay

The pericarp slices bombarded with particle and incubated for 24 h were stored at -20℃ until enzyme extraction. The tissues were ground to a powder in liquid nitrogen and mixed well in extraction buffer consisting of 100mM NaH_2PO_4 (pH7.0), 20mM EDTA, 0.2%(v/v) Triton X-100, 0.2%(v/w) Sarkosyl and 0.07%(v/v) ß-mercaptoethanol. Then the enzyme solution was centrifuged at 15,000 rpm for 10 min at 4℃. Following the determination of protein content in the supernatant (crude extract) using Bradford reagent (Bio-Rad; Bradford,

1976), an equivalent to 20 μg protein was used for the enzymatic reaction for fluorometric assay. The 200 μl of reaction mixture including 80 μl of 2.5 mM 4-methylumbeliferyl glucuronide (MUG) in 50% methanol as a substrate and 120 μl enzyme solution was incubated at 37℃ for 4 h according to Jefferson et al. (1987) and Kosugi et al. (1990). Fluorescence was measured with excitation at 365nm and emission at 455 nm using a spectrophotofluorometer (F2000, Hitachi). The GUS activity was expressed as 4-MU pmol min^{-1} mg $protetin^{-1}$.

Results

Structural characterization of LE-ACS6

The sequence of 5489 nt for *LE-ACS6* was determined and its organization and complete nucleotide sequence is shown in Figure 3.1. The sequence included 2276 bp of 5′-flanking region and 54 bp of 3′-flanking region. It encodes a protein of 477 amino acids, which has all the characteristic features of ACS (Zarembinski and Theologis 1994). The 1431 bp coding region contained four exons which were interrupted by three introns. The 5′- and 3′-untranslated region of the *LE-ACS6* mRNA was 132 and 138 nt long, respectively. In the 5′ flanking region, a putative TATA box sequence (TATTATA) was found at position –58. A potential polyadenylation signal (AATTAT) was located at the position 45 bp downstream from stop codon.

Construction of chimeric genes

To locate the most critical sequences in the promoter regions of *LE-ACS2* and *LE-ACS6* deletions were performed in each promoter, to come up with four fragments each as follows (*LE-ACS2*: -2400, -1493, -695, -308 to –1 bp; *LE-ACS6*: -2211, -1333, -636, -249 to +61 bp). Each fragment was then fused individually to the GUS reporter gene as shown in Figure 3.4.

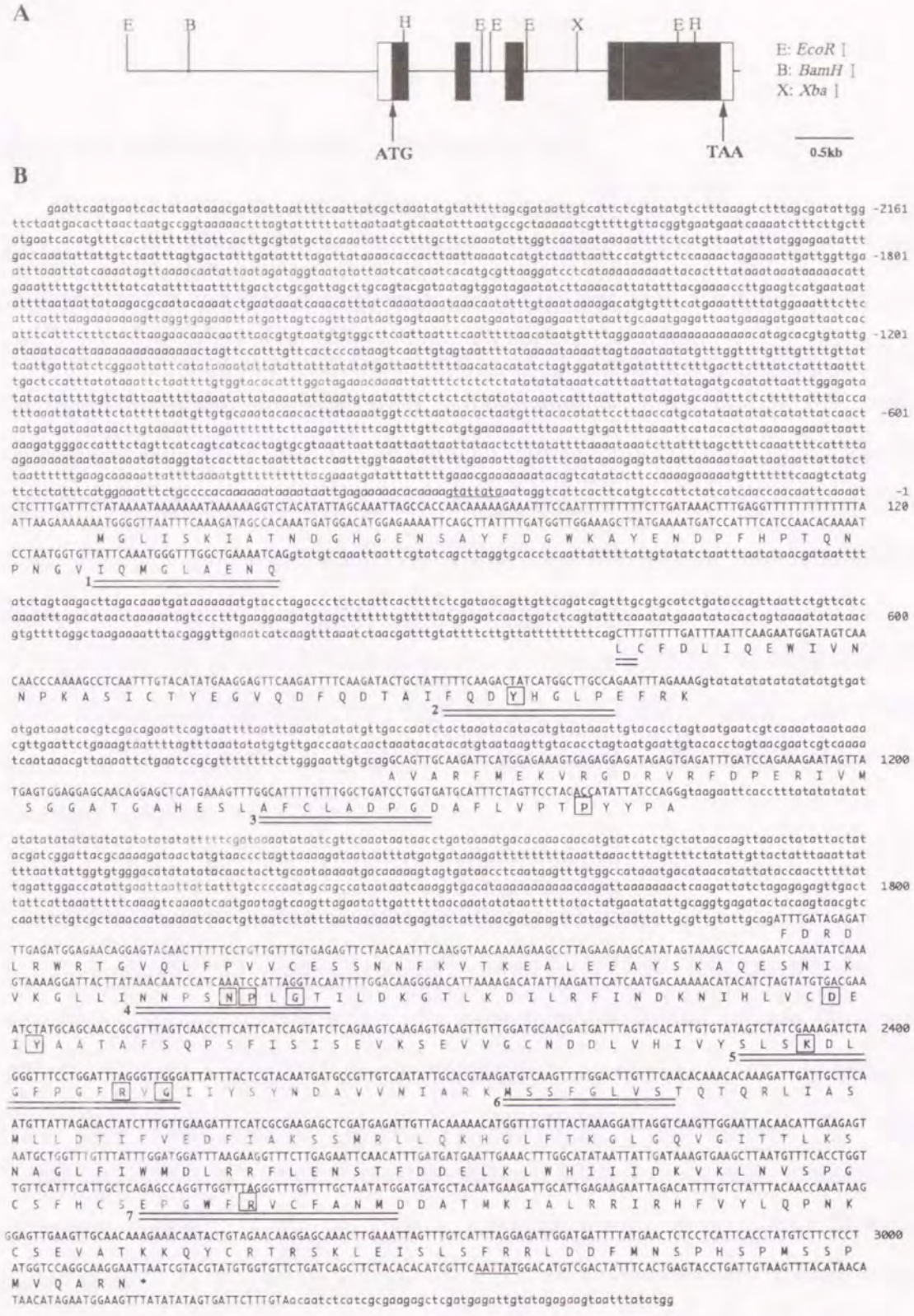


Figure 3.1. Structure of *LE-ACS6* gene. (A) Partial restriction map and gene organization of the *LE-ACS6* gene. The exons are indicated by the filled blocks and the 5′- and 3′-untranslated region are shown as the open blocks. The lines connecting the four exons represent the three introns. Abbreviations for restriction enzymes are as follows; B, *Bam* H Ⅰ; E, *Eco* R Ⅰ; H, *Hind* Ⅲ; X, *Xba* Ⅰ. (B) Genomic DNA sequence of the *LE-ACS6* gene including introns and 5′- and 3′-flanking regions. The nucleotide at position +1 corresponds to the putative transcription initiation site. The sequence upstream from the +1 position are negatively numbered. The mRNA coded by the gene is capitalized, and remainder of the sequence is shown in lowercase. The derived amino acid sequence is presented below the DNA sequence. The 11 boxed residues are invariant amino acids conserved in various aminotrasferase (Kende, 1993; Rottmann et al., 1991). The putative TATA box and polyadenylation signal are underlined.

Response of chimeric genes for propylene and MCP

To confirm whether the longest GUS construct (*LE-ACS2*: -2400 to –1 bp, *LE-ACS6*: -2211 to +61 bp) is enough to confer ethylene sensitivity, GUS transient assay was performed using disc slices prepared from immature (0.16 $nl \cdot g^{-1} \cdot h^{-1}$ producing ethylene) and pink fruit (15.92 $nl \cdot g^{-1} \cdot h^{-1}$ producing ethylene). In immature discs, *LE-ACS2*-GUS activity was not observed in the control (pretreated with MCP) and the activity was strongly induced by propylene. *LE-ACS6*-GUS activity was high in the control and propylene reduced its activity. (Fig. 3.2.). In pink-stage slices, *LE-ACS2* promoter activity was significantly high in the control fruit and MCP dramatically suppressed this activity (Fig. 3.3.). GUS activity for *LE-ACS6* was low in both control and MCP pretreated fruit (Fig. 3.3.). Thus, the GUS constructs both for *LE-ACS2* and *LE-ACS6* had enough length to confer ethylene sensitivity, showing the expected results obtained in Northern analysis in the former Chapter.

Identification of cis-acting element for ethylene response

To determine ethylene-responsive element for positive and negative feedback regulation, GUS transient assay was performed using four deleted constructs. *LE-ACS2*- and *LE-ACS6*- GUS constructs were introduced to pink and immature stage fruit slices respectively. In *LE-ACS2*-GUS chimeric gene, the longest construct (-2400 to –1 bp) showed the highest GUS activity and deletion of the promoter region to –1493 bp had no significant effect (Fig. 3.4A). However, deletion to –695 or –308 bp decreased GUS activity dramatically. *LE-ACS6*-GUS chimeric had high GUS activity in the longest (-2211 to +61 bp) and second longest (-1333 to +61 bp) construct (Fig. 3.4B). The GUS activity decreased significantly by deletion from –1333 to –636 bp and the shortest fusion construct (-249 to +61 bp) showed almost no activity.

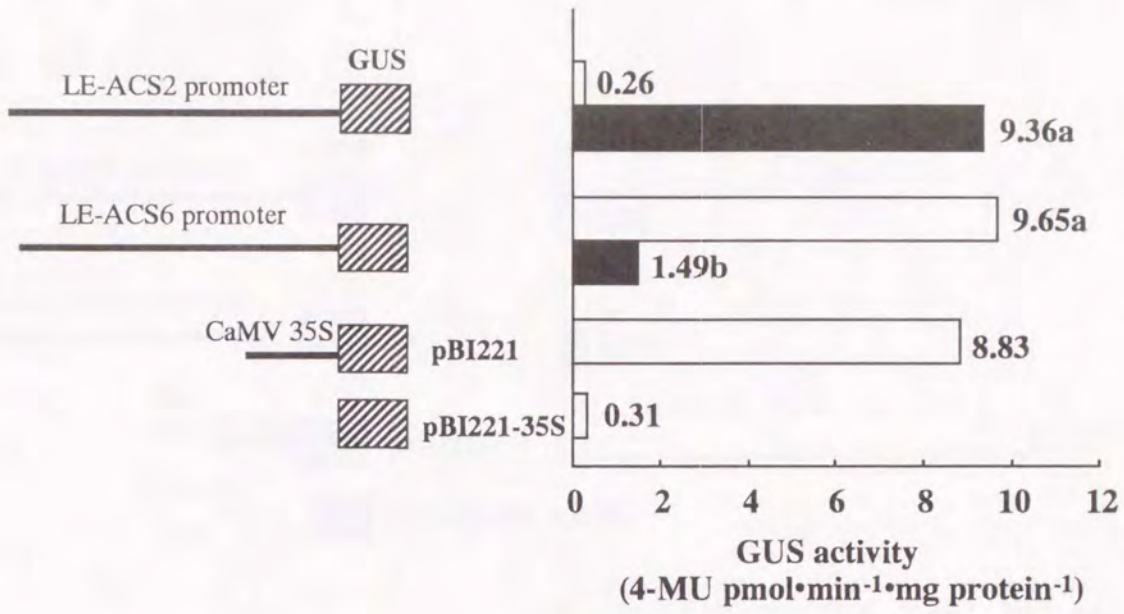


Figure 3.2. Effect of propylene on GUS activity in immature-green fruit slices. Schematic representations of *LE-ACS2* and *LE-ACS6* promoter-GUS chimeric genes (-2400 to -1 bp and -2211 to +61 bp, respectively). CaMV 35S promoter-GUS chimeric gene (pBI221) and only GUS gene (pBI221-35S) are shown on the left with corresponding GUS activites on the right. On the left of the diagram, thin lines represent *LE-ACS2*, *LE-ACS6* or CaMV 35S 5'-flanking sequence. The open box and the closed box shown, are control (pretreated with MCP) and propylene treated slices, respectively. Each determination was carried out with ten replications and the means followed by the same letter was not significantly different by Duncan's LSD at 5% level. GUS activity conferred by pBI221 and pBI221-35S were assayed as positive and negative control, respectively.

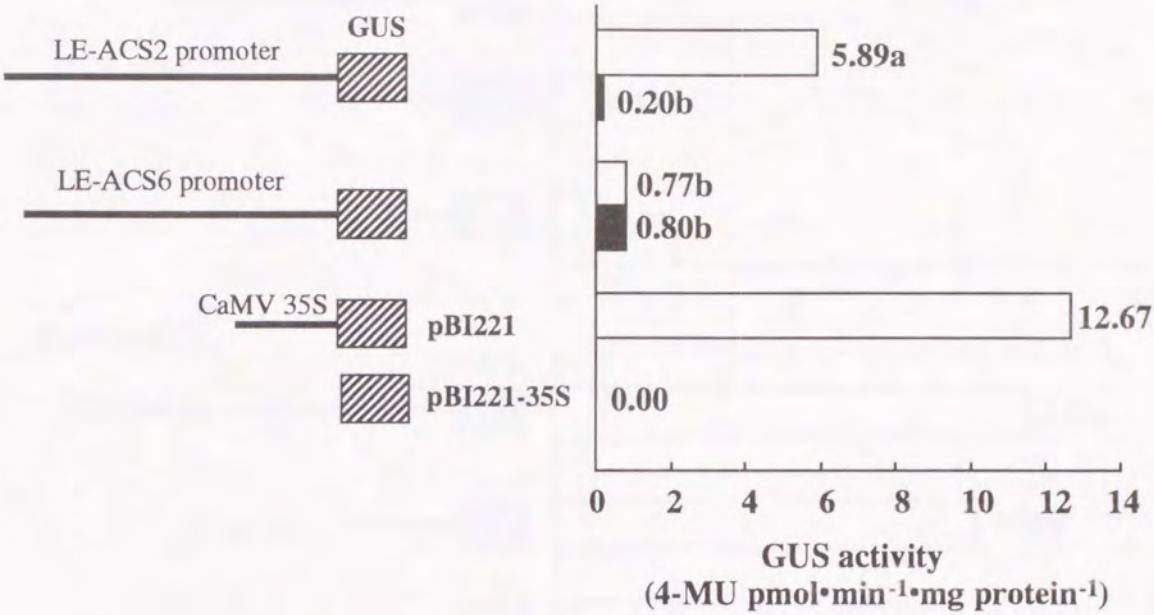


Figure 3.3. Effect of MCP on GUS activity on the fruit slices at the pink stage. The GUS constructs were the same as in Figure 3.2. The open box and the closed box shown, are the control slices and MCP treated slices, respectively. Each determination was carried out with ten replications and the means followed by the same letter was not significantly different by Duncan's LSD at 5% level. GUS activity conferred by pBI221 and pBI221-35S were assayed as positive and negative control, respectively.

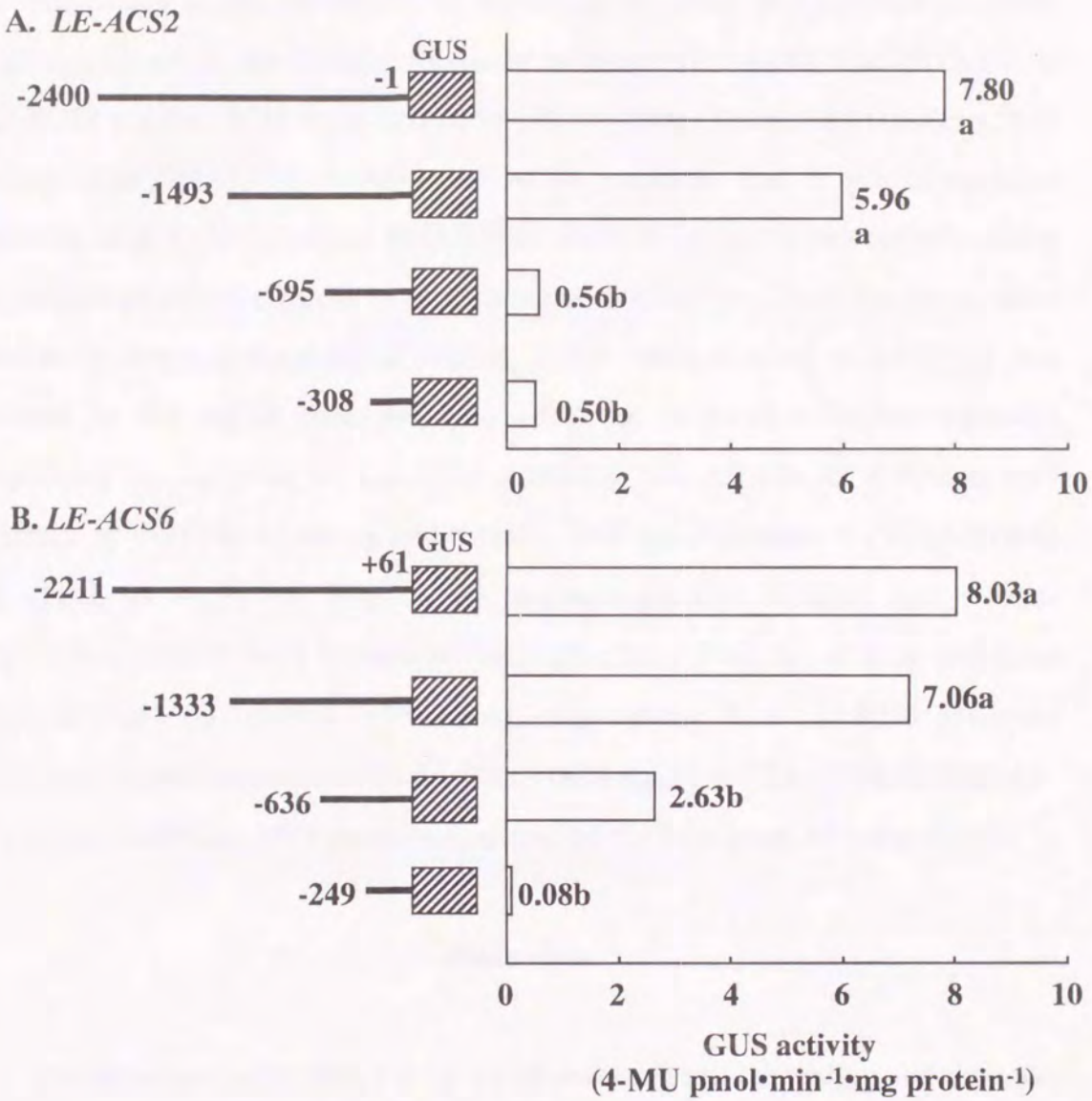


Figure 3.4. Deletion analysis of the *LE-ACS2* and *LE-ACS6* promoter in a transient gene expression system. Assays were carried out using pink (A) and immature-green (B) stage slices. The means of ten replications followed by the same letter was not significantly different by Duncan's LSD at 5% level.

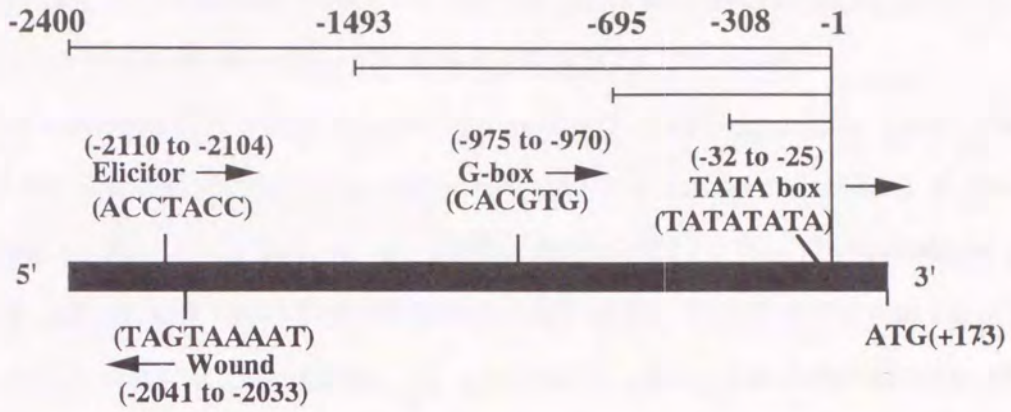
Competition of known cis-element to LE-ACS2 and LE-ACS6 promoter

Figure 3.5 shows the identified *cis*-acting elements in *LE-ACS6* promoter region analyzed in this Chapter compared to those in *LE-ACS2*. The TATA box of *LE-ACS2* and *LE-ACS6* were located in –32 to –25bp (TATATATA) and in –58 to –52bp (TATTATA), respectively. *LE-ACS2* promoter had a wound-response element in 5′-flanking region from –2110 to –2104 bp and a reverse orientation of elicitor-response element in the region from –2033 to –2041 bp. In putative positively ethylene-responsible region, G-box core element (CACGTG) was located in the region from –975 to –970 bp. A putative region regulated negatively by ethylene in *LE-ACS6* promoter was present as a G-box core element in –1211 to –1206 bp and a young fruit specific element (TGTAGTAA) in –1142 to –1135 bp (Fig. 3.5B). Auxin-responsive element and wound-responsive element were located in the region from –469 to –476 bp and from –280 to –287 bp (reverse orientation), respectively. The *LE-ACS6* promoter contained repeat sequence of 33 bp (TATATATAAATCATTTAATTATTATAGAT GCAA) at –889 and –774 position upstream of the start point of transcription.

Discussion

We cloned genomic DNA for *LE-ACS6* and analyzed the coding and promoter regions so as to understand their transcriptional regulation. Fluhr and Mattoo (1996) indicates that *ACS* genes fall into three classes based on the existing number of introns; four intron genes (zucchini, *CP-ACS1A* and *CP-ACS1B*), three intron genes (rice, *OS-ACS1*; *Arabidopsis*,*ACS1*, *ACS2* and *ACS4*; and winter squash, *CM-ACS2*), and two intron genes (potato, *ST-ACS1A*, *ST-ACS1B* and *ST-ACS2*; *Arabidopsis*, *ACS5*; and mung bean, *VR-ACS6*). Among the already cloned tomato *ACS* genomic DNAs, *LE-ACS1A*, *LE-ACS1B*, *LE-ACS2* and *LE-ACS3* have three introns while *LE-ACS4* and *LE-ACS7* include two

A. *LE-ACS2*



B. *LE-ACS6*

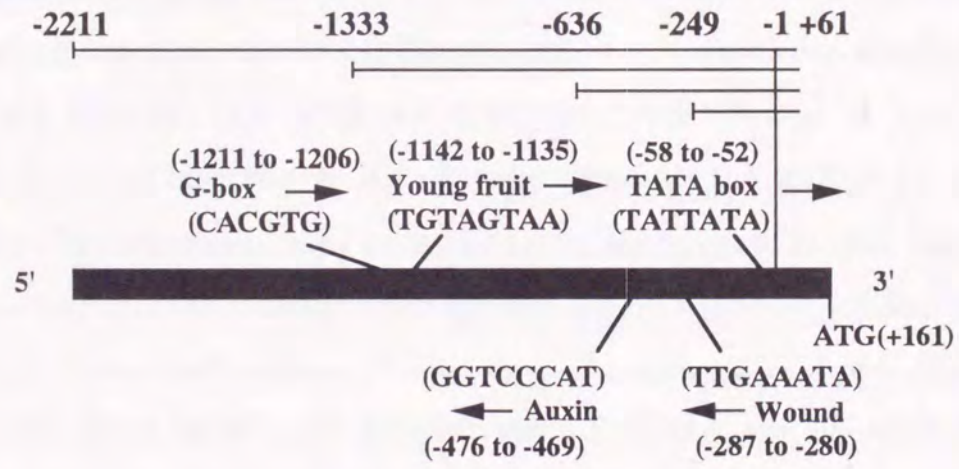


Figure 3.5. Identification of cis-acting regulatory elements in *LE-ACS2* and *LE-ACS6* promoters. Motifs with significant homology to known cis-elements are named as elicitor (Palm et al., 1990), G-box (Williams et al., 1992), wound (Siebertz et al., 1989), young fruit (Carrasco et al., 1993), and auxin (Ballas et al., 1993). The arrow indicates the direction of each element.

introns (Rottmann et al., 1991; Shiu et al., 1998). *LE-ACS6* cloned in the present study had three introns. Number of introns has also been known to differ even in twin genes; for example*CP-ACS1A* and *CP-ACS1B* in zucchini, *ST-ACS1A* and *ST-ACS1B* in potato and *LE-ACS1A* and *LE-ACS1B* in tomato.

The tomato *ACS* genes are phylogenetically subdivided into three classes based on the amino acid sequences between the conserved blocks 4 and 6, (Oetiker et al., 1997; Shiu et al., 1998). According to this classification, *LE-ACS1A*, *LE-ACS1B* and *LE-ACS6* belong to a class Ⅰ, *LE-ACS2* and *LE-ACS4* present in a class Ⅱ, and a class Ⅲ contains *LE-ACS3*, *LE-ACS5* and *LE-ACS7*. Therefore, *LE-ACS2* and *LE-ACS6*, the target genes in this study, belong to different classes. However, Oetiker et al. (1997) suggest that the elicitor inducibility of the seven tomato *ACS* genes (except *LE-ACS7*) is not correlated with their phylogenetic relationship because each class contains one member that is strongly inducible (*LE-ACS2*, *LE-ACS5* and *LE-ACS6*) and at least one member that is not induced (*LE-ACS1A*) or constitutively (*LE-ACS1B*, *LE-ACS3* and *LE-ACS4*) expressed. Similar to elicitor response, positive and negative feedback regulated and constitutively expressed *LE-ACS* genes in tomato fruit is not related to the classification of coding region sequences in the former Chapter. Tatsuki and Mori (1999) also reported that *LE-ACS1A* and *LE-ACS6* were inducible by touch and wound stimuli with rapid and transient expression in both leaves and fruits. As shown in the former Chapter, these two *ACS* genes showed different expression pattern in ripening tomato. Therefore, it is difficult to discuss the differences in the characteristics between *LE-ACS2* and *LE-ACS6* from only the point of view of the sequence of their coding regions.

As a next step in understanding the transcriptional regulation, 5´-flanking regions of *LE-ACS2* and *LE-ACS6* were analyzed using GUS transient assay. The activity of *LE-ACS2* promoter was not detected in young fruit slices while propylene greatly enhanced it. In ripening fruit slices, high activity of *LE-ACS2*

promoter was strongly suppressed by MCP. On the contrary, *LE-ACS6* promoter showed high activity in young fruit slices and this was greatly reduced in the slices pretreated with propylene. The activity of *LE-ACS6* promoter was low in ripening fruit slices irrespective of MCP treatment. These results of GUS transient assay suggest that the promoter activities of both *LE-ACS2* and *LE-ACS6* well reflect the accumulation of their mRNA levels in intact tomato fruit (Chapter 2.2.). Therefore, we considered that, positive and negative feedback regulatory feature of *LE-ACS2* and *LE-ACS6* genes is mainly attributed to the sequences of their promoter regions. However, *LE-ACS6* promoter activity was not detected in the slices prepared from MCP-treated pink stage fruit, in which the accumulation *LE-ACS6* mRNA was recovered from elimination by the production of ripening-ethylene. This contradiction should be resolved in the future.

In order to define ethylene-responsive *cis*-element in *LE-ACS2* and *LE-ACS6* promoter, we carried out deletion analysis with four fragments for each gene (*LE-ACS2*: -2400, -1493, -695 and –308 to –1 bp; *LE-ACS6*: -2211, -1333, -636 and –249 to +61 bp). The *LE-ACS6* promoter activity did not decrease significantly with the deletion to –1333 bp but strong reduction was observed in the construct deleted to –636 bp. The *LE-ACS2* promoter activity showed similar pattern to that of *LE-ACS6* and deletion to –695 decreased the activity. These results suggest that there is a regulatory *cis*-element from –1333 to –636 bp in *LE-ACS6* promoter and from –1493 to –695 bp in *LE-ACS2* promoter.

Ethylene-regulated gene transcription has been studied in detail from three different concepts (Deikman, 1997). First, in promoter region of pathogenesis-related (PR) protein genes, chitinase or ß-1,3-glucanase and an existence of GCC box (TAAGAGCCGCC) has been identified as an ethylene responsive element (Hart et al., 1993; Ohme-Takagi and Shinshi, 1995; Shinshi et al., 1995). Deikman (1997) suggest that different mechanisms must exist to bring about

ethylene-responsive transcription of these genes because GCC box has not been identified in the genes related to flower senesce or fruit ripening, including *LE-ACS2* of tomato plant. In the present study, GCC box was not found in *LE-ACS6* promoter region.

As for the second concept, it is known that the transcript of glutathione S-transferase (GST) gene increases concomitant with flower senescence caused by ethylene in carnation. In this gene, it has been pointed out that essential *cis*-acting elements are located in the region from –667 to –470 bp upstream of the transcription start site and ethylene responsive element binding protein recognizes the region from –510 to –488 bp (Itzhaki et al., 1994). Interestingly, 8 bp sequences (ATTTCAAA) in *GST1* promoter region are similar to the sequence from the promoter of the ethylene-responsive fruit ripening gene, *E4* in tomato (Deikman, 1997). From these facts, Deikman (1997) suggested the possibility that the genes regulated in flower senescence and fruit ripening, may have a common feature. The third concept has been shown on the ethylene-inducible *E4* and *E8* genes in tomato fruit. For ethylene response, *E4* requires two *cis*-elements that act cooperatively. The upstream regulatory element is localized between –150 and –121 bp, and the downstream regulatory element is present from –40 to +65 bp (Xu et al., 1996). In the case of *E8*, sequences from –1528 to –1100 bp are necessary and sufficient for ethylene response in tomato fruit (Deikman et al., 1998). *LE-ACS6* promoter region cloned in this study did not contain the sequences of AA/TTTCAAA that was identified to be necessary for ethylene response in carnation *GST1* and tomato *E4* genes. However, this element is present in three copies in the *LE-ACO1* promoter between nucleotides –473 and –1662 (Blume and Grierson, 1997) and in one copy in apple *ACO* position –1799 (Atkinson et al., 1998). These evidences suggest that this element may be important for ACO transcription.

In the present study, although both *LE-ACS2* and *LE-ACS6* genes had

sequences resembling the three *cis*-acting elements indicated by Deikman (1997), these promoters contained the G-box core element in the expected ethylene-regulatory regions. The G-box is located at –975 and –1211 bp in *LE-ACS2* and *LE-ACS6* promoters, respectively. There may be a possibility that the G-box plays an important role in the ACS transcription because, promoter activity decreased significantly in both *LE-ACS2* and *LE-ACS6* when their GUS-constructs were deleted, so as to exclude the G-box sequences.

Lasserre et al. (1997) indicated that *CM-ACO1* promoter region is responsible for internal or external stimuli including ethylene, wound, pathogen attack and heavy metal in transgenic tobacco with many stress-response elements homologous to other genes. Interestingly, *LE-ACS6* promoter contained young fruit specific element. Similar to *CM-ACO1*, there must be many specific sequences that could recognize internal/external various stimuli in *LE-ACS2* and *LE-ACS6* promoter. In future, it will be necessary to carry out the detailed deletion analysis in *LE-ACS2* and *LE-ACS6* promoter regions.

Summary

In order to investigate the transcriptional regulation mechanism of *LE-ACS2* and *LE-ACS6* that were regulated in opposite feedback directions by ethylene, their promoter activities were determined using GUS transient assay in tomato fruit slices. Four promoter-GUS fusion genes were prepared having different sequence lengths, both for *LE-ACS2* (-2400, -1493, -695 and –308 to -1 bp) and *LE-ACS6* (-2211, -1333, -636 and –249 to +61 bp). In the immature fruit discs, *LE-ACS6* promoter (-2211 bp) showed high activity, while, propylene, ethylene analog, strongly inhibited the activity. In the pink-stage fruit slices, GUS activity driven by *LE-ACS6* promoter was low and not affected by MCP. GUS activity conferred by *LE-ACS*2 promoter (-2400 bp) was low but was strongly induced by propylene in immature-stage fruit slices. In pink-stage fruit slices, high activity of *LE-ACS2* promoter was strongly suppressed by MCP. The promoter activity dramatically decreased by deletion to –695 and –639 bp in *LE-ACS2* and *LE-ACS6*, respectively. These results suggest that an enhancer element may be located from –1493 to –695 bp and from –1333bp to –636 bp in *LE-ACS2* and *LE-ACS6* promoter region, respectively. Within this enhancer region, G-box core element (CACGTG) was present in both gene promoters. The promoter of *LE-ACS6* contained young fruit specific element.

Chapter 4. Expression of *E4* and *E8* genes in the fruit during development and ripening in relation to the action of ethylene

Introduction

Fruit ripening requires the action of a large number of specific enzymes, which contribute to the alteration of texture, flavor, color and chemical compositions. These changes are triggered by the burst of ethylene in climacteric fruits (Yang, 1985). To date, several attempts have been made to isolate ripening specific genes employing molecular techniques such as differential display methods (Slater et al., 1985). Among ripening specific genes isolated so far, *E4* and *E8* are genes that were cloned based on ethylene specific expression during ripening in tomato fruits (Lincoln et al., 1987). *E8* gene is also identical to *pTOM99* gene, a ripening-related gene, obtained from tomato using a differential hybridization technique (Gray et al., 1992; Slater et al., 1985). The expression of *E4* and *E8* genes has been shown to increase within 30 min after the treatment of ethylene, for tomato fruits at mature green stage, and its accumulation was very high during the ripening stage (Lincoln et al., 1987). It has also been found that treatment of intact mature green fruit with NBD, an inhibitor of ethylene action, inhibited both ripening and the expression of *E4* and *E8* genes when compared with control fruits exposed to air (Lincoln et al., 1987), suggesting that the expression of these two genes is regulated by ethylene. However, *E8* is also expressed in transgenic tomato, which has an *ACS* antisense gene and shows reduced ethylene biosynthesis (Theologis et al., 1993). Moreover *E8* is transcribed at reduced but still significant levels in the *Never-ripe* (*Nr*) tomato fruits, which is defective in ethylene perception due to the mutated *NR* gene, a tomato homolog of *ETR* gene family (Wilkinson et al., 1995). These findings led to the concept that although these genes are ethylene responsive, they may also

respond to other ripening signals.

Since the *E4* and *E8* genes are expressed at a high level during fruit ripening and are transcriptionally activated by ethylene, the promoters of these genes have gained lots of attention for potential use in expression of foreign genes during fruit ripening. The promoter activities have been investigated by using fusion genes between 5′ upstream region of the two genes and reporter genes such as GUS and luciferase (LUC), and several *cis*-elements have already been identified in the 5′ upstream regions (Deikman et al., 1998; Montgomery et al., 1993; Xu et al., 1996).

Although the function of *E4* and *E8* are still unknown, the predicted polypeptide encoded by *E4* has a homology to a peptide of methionine sulfoxide reductase from *E. coli* (Montgemery et al., 1993). In addition, *E8* encodes a protein with a sequence homology to iron (Ⅱ) dependent dioxygenase family, encompassing ACO, which plays an important role in regulating ethylene biosynthesis (Deikman and Fischer, 1988). In order to understand the role of *E8*, transgenic plants, which have*E8* antisense or sense gene and reduced *E8* transcription, were analyzed and showed increased ethylene level in ripening fruit. Their reports suggest that the product of the *E8* gene may negatively regulate ethylene biosynthesis during fruit ripening (Kneissl and Deikman, 1996; Penarrubia et al., 1992).

In this Chapter, as a final study on the internal feedback regulation of ethylene biosynthesis in tomato fruit, the expression of *E4* and *E8* genes was more precisely characterized using MCP, an inhibitor of ethylene action, and propylene, an ethylene analogue.

Materials and Methods

Plant materials and treatments

Greenhouse-grown tomato (*Lycopersicon esculentum* Mill. cv Momotaro) fruit were harvested from a commercial farm. Determination of developmental stage in tomato fruit and treatments with MCP and propylene were carried out as previously described (Chapter 2.2.). Mature green fruit were divided into three stages based on basal level of ethylene production: MG1, MG2, and MG3. After measurement of ethylene production, pericarp tissues from the fruit equatorial region were frozen in liquid nitrogen and stored at -80℃ until RNA extraction.

Measurement of ethylene production

Ethylene production from ripe fruit was measured by incubating samples in an airtight chamber for 1 h at 22℃, withdrawing 1 ml of headspace gas from the chamber, and injecting into a gas chromatograph (Chapter 2.2.). For immature and mature green fruits, the basal level of ethylene production was measured by the method of Akamine and Goo (1978), as described in Chapter 2.2.

RNA extraction, cloning and sequencing

RNA was extracted by the hot borate method (Wan and Wilkins, 1994) from ripe tomato fruit. Poly $(A)^+$ RNA was isolated using Oligotex-dT30 (Takara, Kyoto, Japan) according to the manufacturer's protocol. The first-strand cDNAs synthesized by reverse transcriptase (RT) from 2 μg of poly $(A)^+$ RNA were used as template for RT-PCR with specific primers A (bp 1460-1483) and B (bp 2028-2050) with restriction site sequence of *Eco*R I for *E4* (accession no. S44898) and primers C (bp 1171-1195) and D (bp 2121-2143) with restriction site sequence of *Eco*R I for *E8* (accession no. X13437), which were designed from nucleotide sequences registered on databases (Table 4.1.). The PCR products were ligated

Table 4.1. Oligonucleotide primers used for amplification of cDNAs by RT-PCR.

Name		DNA sequence	gene
A	E4-F	5´-g(gaattc)cagcaagtcaaccaccaatccagc-3´	E4
B	E4-R	5´-g(gaattc)ccgactgcttacaacctctgccc-3´	
C	E8-F	5´-g(gaattc)cagcgtttgatgatactaaggccgg-3´	E8
D	E8-R	5´-g(gaattc)ccgagaccgagaccttcagacaa-3´	

into pGEM plasmid vector (Promega) and then introduced into *E. coli* JM109. Target cDNAs were sequenced using a DNA sequencer (ABI PRISM 377, Applied Biosystems).

The method of RNA blotting and hybridization were identical to those described in Chapter 2.1.

Results and Discussion

Isolation and identification of E4 and E8 gene fragments

E4 and *E8* are genes which were screened by the substractive hybridization technique, in which a probe enriched for ethylene-inducible sequence from tomato fruit cDNA library was hybridized with the early ripening stage specific sequence (Lincoln et al., 1987). *E8* is identical to *pTOM99*, a ripening-related gene, obtained from tomato using a differential hybridization technique (Gray et al., 1992; Slater et al., 1985). To obtain probes for Northern hybridization, we cloned two fragments of *E4* and *E8* genes from ripening tomato fruit using specific oligonucleotide primers. Nucleotide sequence of each fragment was completely identical to those of corresponding cDNAs previously registered in the database: *E4* (accession no. S44898); and *E8* (accession no. X13437).

In order to characterize minutely the expression of *E4* and *E8* genes in relation to onset of ripening, we collected fruits from mature green to turning stages, all of which had different levels of basal ethylene (Fig. 4.1). Ripening ethylene production (system 2 ethylene) began at the MG2 stage. The abundance of *E4* mRNA was detectable at the MG1 stage and then increased toward the turning stage. In contrast, the *E8* transcript, which was undetectable at the MG1 stage, appeared at MG2 stage and then increased gradually as the rate of ethylene production increased. The result showed that the expression of *E4* and *E8* gene

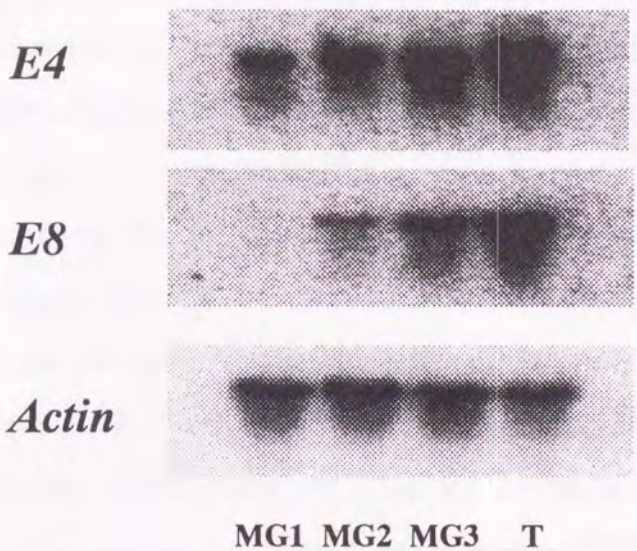


Figure 4.1. Changes in the accumulation of mRNAs corresponding to *E4* and *E8* genes in fruit with different rates of ethylene production from the mature green stage to the turning stage. MG1 fruit with 0.18, MG2 fruit with 0.36, MG3 fruit with 0.96, and turning (T) fruit with 1.46 $nl \cdot g^{-1} \cdot h^{-1}$ of ethylene production were used. Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amount of mRNA loaded.

are associated with fruit ripening in a different manner.

Regulation of the expression of E4 and E8 by ethylene

The expression of *E4* and *E8* have been characterized by several researchers and shown to be an ethylene responsive. Lincoln et al. (1987) demonstrated that the abundance of *E4* and *E8* mRNAs were enhanced by exogenous ethylene within 30 min in tomato fruit at mature green stage and an inhibitor of ethylene action, NBD, inhibited both fruit ripening and the increase of the abundance of these mRNAs in mature green fruit. The enhancement of *E4* and *E8* gene expression by ethylene is due to transcriptional activation (Lincoln and Fischer, 1988a). *E4* gene transcription is activated by ethylene in both leaves and fruit, whereas *E8* gene transcription is strongly activated in fruit, but not in leaves (Lincoln and Fischer, 1988a). However, even through, *E4* and *E8* gene expression is enhanced by ethylene and inhibited by an inhibitor of ethylene action, it is still not sufficient to conclude that the expression of these genes are directly controlled by ethylene, because ethylene can induce multiple effects on fruit ripening, one of which might enhance the gene expression as a secondary effect. In order to verify whether ethylene directly activates the accumulation of the mRNAs or not, we applied a strong inhibitor of ethylene action, MCP (Serek et al., 1994), to tomato fruit at turning and pink stages when these mRNAs had accumulated at sufficienty high level.

Figure 4.2. shows the changes in ethylene production and expression of *E4* and *E8* genes in tomato fruit during development and ripening. *E4* gene was already expressed at limited level in preclimacteric fruit (IM and MG stages) which produced only basal ethylene, and its abundance increased further upon commencement of ripening. The accumulation of the *E4* mRNA declined to a large extent by treatment of fruit with MCP at either turning or pink stage. In particular, 2 days after MCP treatment, the *E4* mRNA expression almost

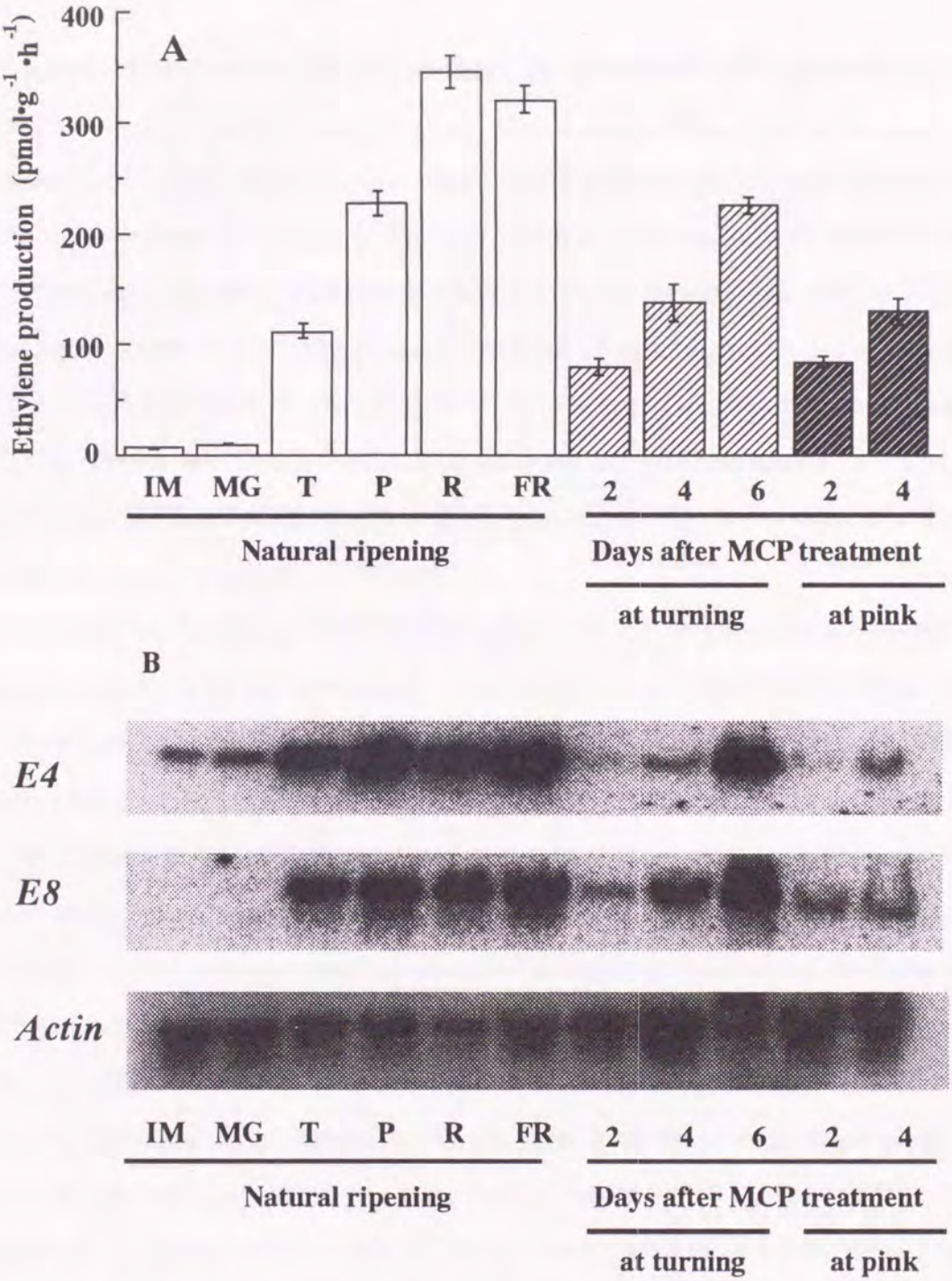


Figure 4.2. Ethylene production and expression of *E4* and *E8* genes in tomato fruit during development and ripening, and effect of MCP. (A) Fruit harvested at turning and pink stages were treated with 10 to 20 nl•liter^{-1} MCP for 6 hours and then ripened at 22℃. (B) mRNAs were prepared from the fruit immediately after the determination of ethylene levels. Each lane in natural ripening fruit shows immature green (IM), mature green (MG), turning (T), pink (P), red (R), and full-ripe (FR) stages. Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amount of mRNA loaded.

completely disappeared. This suppression by MCP recovered gradually in the next 2 and 4 days. On the other hand, the abundance of *E8* mRNA in the fruit was undetectable at the preclimacteric stage and increased concomitantly with the burst of ethylene production. The *E8* mRNA abundance was considerably decreased by treatment of fruit with MCP at both the turning and pink stage, and afterwards, recovered to control level. MCP has been thought to bind to ethylene receptor and inactivate it, resulting in elimination of the effects of endogenous ethylene (Sislar and Serek, 1997). The decrease of the abundance of *E4* and *E8* mRNAs by MCP treatment confirmed the concept that the expression of *E4* and *E8* gene are up-regulated by ethylene.

In order to determine the response of *E4* and *E8* to exogenous ethylene in immature fruit, the fruit was treated with propylene at 5000 nl•liter^{-1}, equivalent to 50 nl•liter^{-1} ethylene (Burg and Burg, 1967), for 2 and 4 days (Fig. 4.3.). There was no increase in ethylene production in the fruit from the basal level and the accumulation of *E4* mRNA was unaffected by the propylene treatment. This observation does not necessarily imply that *E4* gene is not regulated by ethylene, since the same treatment does not induce the expression of ethylene-inducible *ACS* genes (Chapter 2.2.). Indeed, *E8* gene expression was induced by propylene treatment (Fig. 4.3.). Furthermore, the mRNA for *E4* and *E8* genes have been shown to accumulate to similar levels in both ethylene treated *rin*, a ripening inhibited mutant, and wild type tomatoes (Lincoln and Fischer, 1988b). This suggests that higher concentration or longer time treatment with ethylene might be needed to induce the increase of *E4* transcript in such young fruit.

In the promoter analysis of *E4* and *E8* genes, several *cis*-elements and *trans*-acting factors have been identified (Cordes et al., 1989; Coupe and Deikman, 1997; Deikman et al., 1992, 1998; Montgomery et al., 1993; Xu et al., 1996). Two cooperative *cis*-elements in the promoter region of *E4*, at least, have been shown to be required for ethylene-responsive transcription (Xu et al., 1996). The

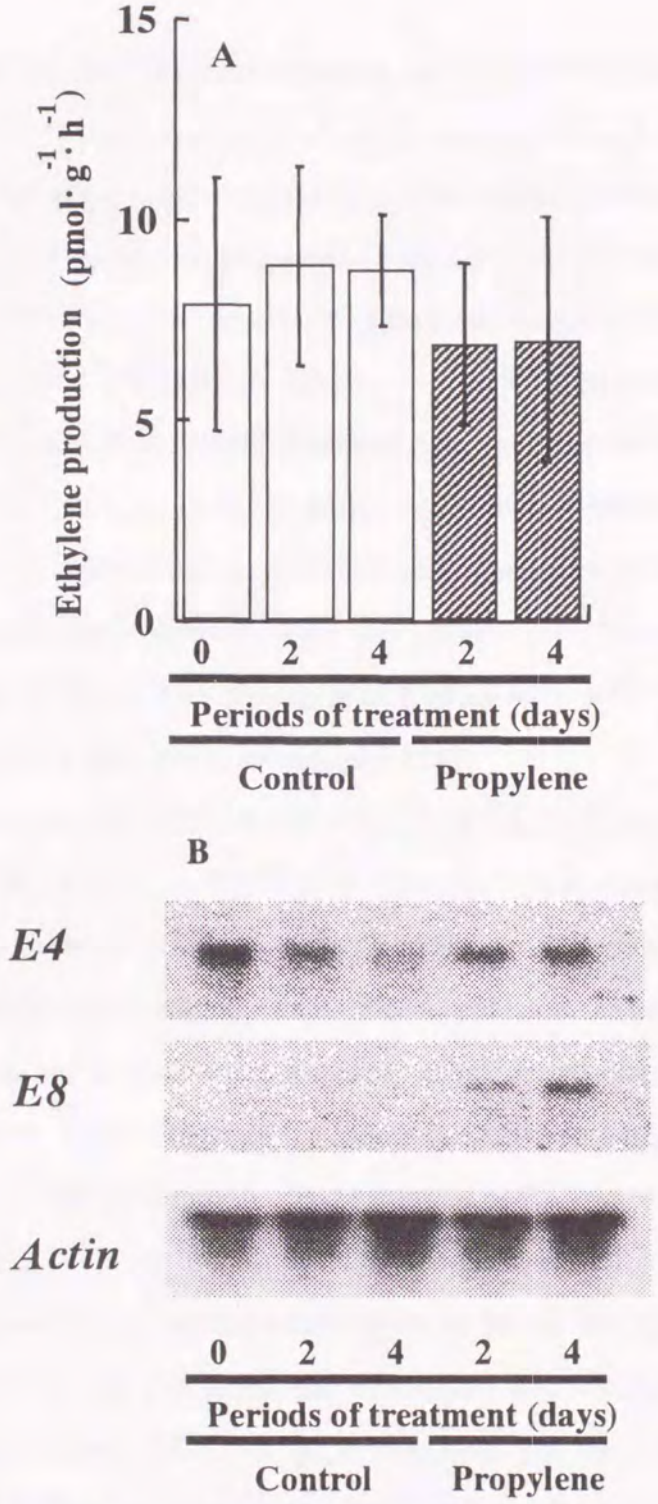


Figure 4.3. Effect of propylene on the ethylene production and the accumulation of mRNAs corresponding to *E4* and *E8* genes in immature green fruit. (A) Fruit were harvested about 2 weeks after flowering, and then treatment with 5000 μl•liter^{-1} propylene for 2 and 4 days at 22℃. (B) Each lane contained 3 μg of mRNA. Actin was used as an internal control to normalize the amount of mRNA loaded.

model proposed is that two *trans*-factors, named E4/E8BP and E4-UpEREBP, interact with the two *cis*-elements in *E4* gene. Interestingly *E4* and *E8* genes share two similar sequences recognizing the two DNA binding proteins (Coupe and Deikman, 1997). In the promoter region of *E8*, the *cis*-element for E4-UpEREBP is located in the domain required for ethylene response, while the other *trans*-element, E4/E8BP is included in the domain for organ specific expression (Deikman et al., 1998). Furthermore, one other set of *cis*-element and *trans*-factor in *E8* gene has been identified and shown to be involved in ripening, but has an ethylene-independent transcription. The present results agreed with this model, in that the expression of *E8* was induced by ethylene, but remained at significant level even in the ripening fruit treated with MCP, which completely inactivated ethylene receptor present in the fruit.

On the other hand, *E4* mRNA accumulated at low level even in immature and mature green fruits (Fig. 4.3.). Up to now, no *cis*-element nor *trans*-factor associated with these stages have been identified in the promoter region of *E4*. Two alternative mechanisms may explain the expression. The first hypothesis is that, the expression is supported by basal ethylene produced at these stages, based on the assumption that *E4* is hyper-sensitive to ethylene. This can be supported by the almost complete disappearance of *E4* transcript in the ripening fruit treated with MCP in this study. The second one is that the expression is controlled by another developmentally-regulated factor in young fruit. To verify the two hypotheses, we compared the abundance of *E4* mRNA in young fruit treated with or without MCP. As MCP treatment did not affect the low level expression of *E4* (data not shown), we concluded that the expression is due to an unknown factor independent of ethylene. Further promoter analysis might be needed to establish the molecular mechanism of *E4* gene expression.

Role of E8 toward ethylene signaling

It is known that E8 protein negatively regulates ethylene biosynthesis in tomato fruit because ethylene production increases in antisense transgenic plant (Penarrubia et al., 1992). However, E4 protein function is unclear and its protein is not necessary for the ripening process because *LE-ACS2* antisense fruit treated with propylene ripen normally in the absence of E4 gene expression (Theologis et al., 1993). Chapter 2.2. showed that *LE-ACS6* is negative regulated by ethylene. *LE-ACS6* and *E8* expression indicate an opposite pattern in both preclimacteric and climacteric tomatoes. When accumulation of one gene mRNA is detected, another gene transcript is absent. The results may support that *E8* is a component of negatively ethylene signaling. However, it is unclear whether autoinhibitory mechanism directly or indirectly mediates E8. In future, *ETR* gene family and *E8* double mutant analysis may provide additional information on feedback regulation mechanism by ethylene.

Summary

We characterized the expression of *E4* and *E8* genes during development and ripening associated with ethylene in tomato fruit. In preclimacteric fruit which produced basal ethylene, only *E4* gene was detectable. As maturity progressed, abundance of *E4* and *E8* mRNAs increased concomitantly with the burst of ethylene production. Inhibitor of ethylene action, MCP, suppressed accumulation of these transcripts at both the turning and pink stages. In particular, *E4* mRNA was almost completely eliminated 2 days after MCP treatment. On the other hand, propylene treatment induced only accumulation of *E8* mRNA. These results indicate that the expression of *E4* and *E8* are strongly regulated by ethylene in ripening fruit and suggest that *E4* gene is controlled by an unknown ethylene-independent factor in unripe fruit.

Chapter 5. General Discussion and Conclusion

Ethylene has been shown to regulate its own biosynthesis in two opposite directions. In positive feedback regulation, ethylene stimulates its own synthesis, and in negative feedback regulation, ethylene inhibits its own synthesis (Yang and Hoffman, 1984). At the gene expression level, the involvement of a positive feedback regulation in ethylene biosynthesis has been elucidated in ripening fruit, namely for *ACS* and/or *ACO*, the two key enzymes in the ethylene biosynthetic pathway. In tomato fruit, ethylene production during the climacteric stage has been demonstrated to be due to the accumulation of transcripts of two *ACS* genes, *LE-ACS2* and *LE-ACS4* (Lincoln et al., 1993; Rottmann et al., 1991), and one *ACO* gene, *LE-ACO1* (Barry et al., 1996). In addition, it has been demonstrated that exposure of mature green fruit to exogenous ethylene induces transcription of the above two *ACS* genes in a dose-dependent manner (Lincoln et al., 1993). Liu et al. (1985) reported that the low level of ACO activity in mature green tomato fruit increased markedly upon ethylene treatment, in a dose- and time-dependent manner and that this increase is inhibited by NBD, an ethylene action inhibitor. Therefore, it is likely that *ACO* gene expression is also regulated under a positive feedback system. Thus, although the positive-regulated features of *ACS* and *ACO* have been demonstrated in preclimacteric tomato as mentioned above, it has not yet been clarified whether or not the same regulation system operates in the fruit even after autocatalytic burst ethylene production. In the present study, this was elucidated initially in Chapter 2.1. In the fruit ripened at the turning stage, ethylene production and activities of both ACS and ACO increased concomitantly with the increased abundance of *LE-ACS2*, *LE-ACS4* and *LE-ACO1* mRNAs as maturity progressed. These increases with ripening were prevented to a large extent by treatment with the ethylene action inhibitor, MCP. These results demonstrate that a strong positive feedback regulation is

involved in ethylene biosynthesis in the tomato fruit even at the stage of a massive ethylene production. However, expression of two *LE-ACS* genes was completely eliminated 2 days after MCP treatment in turning fruit, but ethylene biosynthesis in the same fruit was not inhibited to the level expected with respect to suppression of the gene expression. This result suggested the existence of another *LE-ACS* gene regulated by negative feedback mechanism.

As the next step in this study, this negatively regulated *ACS* gene was cloned and denoted as *LE-ACS6* gene and its expression characterized in Chapter 2.2. Negative feedback regulation of ethylene biosynthesis has been reported to be wound- and auxin- inducible, in various plant organs and in ethylene receptor mutant plants including the transgenic petunia flowers (Wilkinson et al., 1997), the leaves of *Arabidopsis* (Bleecker et al., 1988) and *Never ripe* tomato (Lund et al., 1998). Transcript of *LE-ACS6* gene accumulated in preclimacteric fruit but was eliminated in ripening fruit. The elimination was recovered by treatment with MCP. Furthermore, the accumulation of *LE-ACS6* mRNA in young fruit was eliminated by propylene treatment. These results strongly suggest that the expression of the *LE-ACS6* gene is regulated by a negative feedback mechanism. In Chapter 2.2., the expression of other two *LE-ACS* genes, *LE-ACS1A* and *LE-ACS3*, was investigated in tomato fruit at various stages. These genes were expressed constitutively in the fruit throughout development and ripening, irrespective of treatment with either MCP or propylene, indicating that the expression of these genes is ethylene-independent and constitutive.

McMurchie et al. (1972) introduced the concept of system 1 and system 2 ethylene. System 1 is the basal low rate of ethylene production present in preclimacteric stages and it is inhibited by exogenous ethylene. The basal level of ethylene produced by vegetative tissues and nonclimacteric fruits can also be classified as system 1 (Oetiker and Yang, 1995). System 2 is the high rate ethylene production in autocatalytic manner observed during ripening in

climacteric fruits and in certain senescent flowers (Oetiker and Yang, 1995). McGlasson (1985) suggested that different ACS may be involved in the two systems of ethylene production. The present results (Chapter 2) support this suggestion and demonstrate that ethylene biosynthesis in tomato fruit is regulated by the three different groups of the *ACS* gene family: (a) *LE-ACS2* and *LE-ACS4* are regulated by a positive feedback mechanism, and they are the dominant genes responsible for system 2 ethylene accompanying ripening process in the fruit, (b) the *LE-ACS6* gene is negatively regulated and is responsible for system 1 ethylene present in preclimacteric fruit, and (c) the *LE-ACS1A* and *LE-ACS3* gene transcripts accumulate constitutively throughout fruit development irrespective of the mode of feedback regulation.

Recently, Tatsuki and Mori (1999) demonstrated that the mRNAs of *LE-ACS6* and *LE-ACS1A* accumulate transiently in response to touch stimuli and wounding in both fruit and leaves in tomato, whereas *LE-ACS2* mRNA accumulate only 2 hours after wounding. The transient accumulation of *LE-ACS6* agrees with the concept that, the gene transcription is regulated by negative feedback system as the ethylene produced in the early phase suppresses the further transcription of this gene. Besides that, Tatsuki and Mori (1999) proposed that, the accumulation of *LE-ACS2* at the later phase in response to wounding, is independent of ethylene, based on the observation that *Nr* mutant tomato fruit showed same expression pattern of *LE-ACS2* mRNA in response to wounded stimuli as wild type fruit. Whether LE-ACS6 is induced by touch stimuli or some other unknown factor requires further investigation.

The transition from system 1 to system 2 of *ACS* gene family observed in this study has also been shown in other fruits. For instance, two *ACS* genes, *capacs 1* and *capacs 2*, are differentially expressed during papaya fruit ripening (Mason and Botella, 1997). The *capacs 1* mRNA level is high in mature green fruit and steadily decrease with ripening. However accumulation of *capacs 2* mRNA is

undetectable in mature green fruit and dramatically increases concomitant with fruit ripening. In yet another case, *PE-ACS1* mainly expressed in ripening passion fruit, is induced by ethylene (Mita et al., 1998). High level of *PE-ACS2* mRNA in the preclimacteric passion fruit decreased with ripening. Thus, in tomato, papaya, and passion fruit, similar regulation mechanism for ethylene biosynthesis may be existing.

At present, five homologs of ethylene receptor genes have been isolated from tomato (Lashbrook et al., 1998). In this study, two genes, *LeETR1* and *NR* (*LeETR3*), were cloned. The abundance of *LeETR1* mRNA accumulated constitutively throughout development and ripening irrespective of treatment with either MCP or propylene. Similar results have been reported that, *LeETR1* expression was unaffected by ethylene and silver ions, an ethylene-action inhibitor (Zhou et al., 1996). Wilkinson et al. (1995) indicated that *NR* mRNA in tomato fruit is positively regulated by ethylene in a development-specific manner in ripening fruit and ethylene-treated mature green fruit. In this work, expression of the *NR* gene showed similar trend to that of *LE-ACS4* and *LE-ACO1* genes including elimination or recovery of mRNA accumulation by propylene or MCP (Chapter 2.2.). The expression of *LE-ACS2*, *LE-ACS4* and *NR* transcripts were not inducible in immature green fruit with exposure to propylene for 4 days, indicating a possible lack of a rapid and autocatalytic system for ethylene biosynthesis in young fruit. Therefore, the transition from system 1 to system 2 ethylene production may be controlled by the accumulated level of NR protein which may increase gradually with fruit age. McGlasson (1985) previously came up with the concept that most fruits become increasingly sensitive to ethylene with time after anthesis. Recently, Tieman and Klee (1999) reported the differential expression of *LeETR4* and *LeETR5*. Interestingly, their results indicate that *LeETR4* is expressed at a very high level, accounting for more than 90% of the putative receptor expression in green fruit and approximately 50% of

the putative expression in ripening fruit (Tieman and Klee, 1999). Similar expression pattern has been reported in passion fruit, whereby the level of expression of *PE-ETR1* did not significantly change over the course of ripening (Mita et al., 1998). These findings suggest that ethylene receptor family may have a key function in the transition from system 1 to system 2 ethylene production, with a distinct role in the positive or negative feedback mechanism throughout development and ripening in the fruit.

As a next approach in understanding the transcriptional regulation of ethylene biosynthesis, we analyzed the function of 5´-flanking regions in *LE-ACS2* and *LE-ACS6* gene which were regulated in opposite feedback mechanism (Chapter 3.). Promoter activities of both *LE-ACS6* and *LE-ACS2* gene reflected their mRNAs accumulation pattern in tomato fruit. *LE-ACS6* promoter activity was high in young fruit but propylene strongly suppressed it. High activity of *LE-ACS2* promoter observed in ripened fruit was strongly inhibited by MCP. From the deletion analysis, it was concluded that *LE-ACS2* and *LE-ACS6* promoter had a putative regulatory *cis*-element from -1333 to -636 bp and from -1493 to -695 bp, respectively. To date, ethylene-regulated gene transcription has been studied in detail from the following three different contexts: (a) pathogenesis-related (PR) genes including chitinase or ß-1,3-glucanase which contains ethylene-responsive element GCC box (TAAGAGCCGCC), (b) glutathione S-transferase (*GST*) in carnation petal, and (c) ethylene-inducible genes (*E4* and *E8*) in tomato fruit (Deikman, 1997). The latter two genes have a similar 8 bp sequences (AA/TTTCAAA) in promoter region. In this study, both *LE-ACS2* and *LE-ACS6* promoter region were found to lack GCC box and the sequences of AA/TTTCAAA that have been identified to be necessary for ethylene response. However, G-box core element was found in *LE-ACS6* promoter in the expected ethylene-regulatory region (Chapter 3.). Similar sequences to the G-box core (CACGTG) has been known in several light-

regulated promoters in *Arabidopsis* (*AT-ACC1*), in tomato *LE-ACS2* promoter (Van Der Straeten et al., 1992), and in mung bean *VR-ACS6* (Yoon et al. 1999). Furthermore, deletion of G-box from the GUS-fusion construct for both *LE-ACS2* and *LE-ACS6* resulted a considerable reduction in promoter activity. Therefore, it is possible that G-box play an important role for ACS transcription in tomato fruit.

Among ripening related genes cloned from tomato fruit, *E4* and *E8* are two genes expressed at very high level in ripening fruit, but not in immature and mature fruit. The expression of these genes has been recognized to be dependent on ethylene. Interestingly, the transgenic tomato fruit in which *E8* gene expression is inhibited by its antisense gene or co-suppression, produces ethylene at much higher rate than the wild fruit (Kneissl et al., 1996; Penarrubia et al., 1992). It has been suggested that *E8* is involved in internal regulation of ripening-ethylene in a negative manner. In order to verify whether ethylene directly enhances the accumulation of the mRNA for *E4* and *E8* or not, I treated tomato fruit with MCP at the ripening stage. The abundance of *E4* and *E8* mRNAs increased concomitantly with the burst of ripening-ethylene while MCP treatment decreased the level of these mRNAs. The results confirmed the concept that the expression of *E4* and *E8* gene are up-regulated by ethylene. Besides, the response pattern of *E8* expression to MCP and propylene was similar to that of *NR*, a member of ethylene receptor gene family. Recently the members of the ethylene-receptor gene family have been shown to act as negative regulators of the ethylene signal transduction pathway and to modulate sensitivity of tissues to ethylene (Hua and Meyerowitz, 1998). Thus, the similarity of ethylene expression pattern between *E8* and *NR* deserves attention. The regulatory mechanism of *E8* to ethylene biosynthesis was not fully elucidated, however, further investigation of physiological function of *E8* would provide better understanding of ripening-ethylene biosynthesis.

In conclusion, the results presented here are summarized in Figure 5.1., suggesting that ethylene biosynthesis in tomato fruit is regulated by the three different groups of *ACS* gene family: a) *LE-ACS2* and *LE-ACS4* are the dominant genes responsible for system 2 ethylene production in ripening fruit and their expression is regulated by a positive feedback mechanism, b) the *LE-ACS6* gene is responsible for the low rates of system 1 ethylene production and is negatively regulated in preclimacteric fruit, and c) the *LE-ACS1A* and *LE-ACS3* genes are also responsible for the preclimacteric system 1 ethylene production, and their transcripts accumulate constitutively throughout fruit development irrespective of the mode of feedback regulation.

In tomato fruit, the preclimacteric system 1 ethylene production is mediated by the *LE-ACS1A*, *LE-ACS3* and *LE-ACS6* genes, together with *LE-ACO1* and *LE-ACO4*. Ethylene production shifts to system 2 at the climacteric stage, with a burst accumulation of *LE-ACS2*, *LE-ACS4*, *LE-ACO1* and *LE-ACO4* mRNAs as a result of positive feedback regulation. This transition from system 1 to system 2 ethylene production might be related to the accumulated level of *NR*, *E4* and *E8* mRNAs from the mature green stage to the turning stage, together with the role of G-box core existing in *LE-ACS2* and *LE-ACS6* promoters Further work is needed to clarify the induction mechanism of fruit ripening, especially in relation to the ethylene signaling.

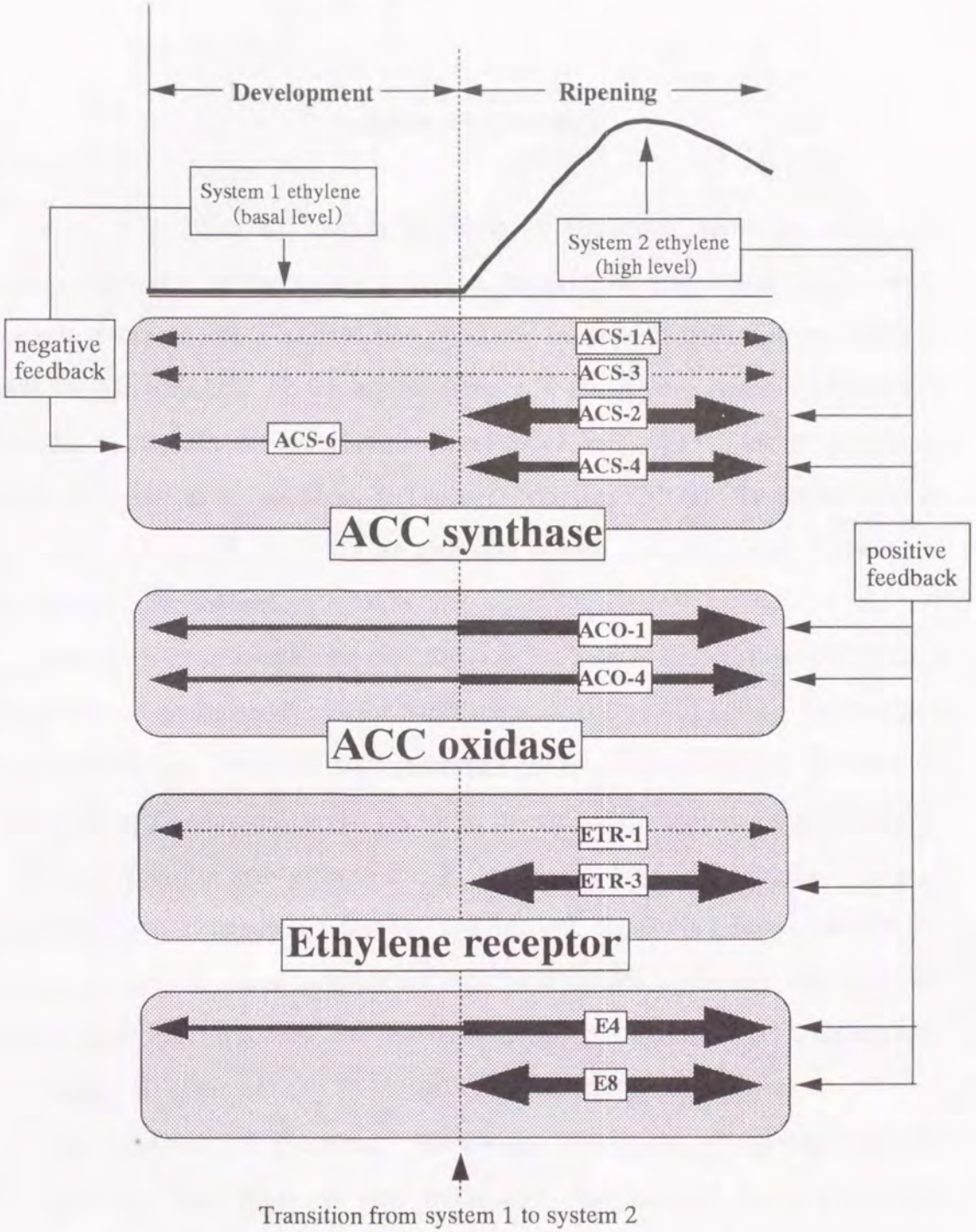


Figure 5.1. Possible mechanism of the internal feedback regulation of ethylene biosynthesis in tomato fruit. Width of the line with arrows indicates the accumulating level of each mRNA in the fruit.

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DECLARATION

It is hereby certified that this thesis is a true record of original research work done by the candidate **Akira Nakatsuka** at the Graduate School of Natural Science and Technology, Okayama University, Japan, and that it has not been submitted previously to any other University either in whole or in part for the award of any degree, fellowship or any other similar titles whatsoever. This thesis is hereby accepted for the award of the **Doctor of Philosophy Degree in Agriculture** of the Okayama University, Japan.

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